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277. VITAMIN A AND C IN COW'S MILK, WITH A NOTE ON THE SYNTHESIS OF VITAMIN C IN BOVINES

BY S. N. RAY, KARAM CHAND
AND K. GOVIND RAU
Imperial Veterinary Research Institute, Muktesar, India

THE estimation of the vitamin contents of milk is of much importance both to the dairy industry and to public health. Nevertheless, only two papers dealing with the estimation of vitamin A in milk and milk products have been published in India (De(1), Grewal & Kochhar(2)). Both deal, however, with the vitamin A content of butter-fat and only indirectly express the vitamin A value of milk. A fairly large volume of milk is necessary to separate out the fat by the usual churning process. We have therefore used a simple method for the estimation of vitamin A in small amounts of actual milk. The method described later in this paper has been found both simple and reasonably accurate.

The number of papers published in India dealing with the vitamin C content of milk has also been surprisingly small considering how simple is the method of determining vitamin C, while the recorded values are very low, e.g. 0·4 mg. ascorbic acid per 100 ml. milk by Ranganathan(3) and 0·7 mg./100 ml. milk by Chakravarty(4). These values compare very unfavourably with the corresponding English figures of 2–3 mg./100 ml. milk (Harris & Ray(5); Bartlett *et al.*(6)). Indian workers have, however, generally worked with milk from the bazaars, so that it is impossible to say whether this low vitamin concentration is an inherent property of the milk of Indian breeds of cattle or whether it is due to the destruction of the vitamin by light during storage (Kon & Watson(7)). For these reasons, it was decided to examine the vitamin C content in samples of milk from several breeds of Indian cattle.

EXPERIMENTAL

All the animals used formed part of the dairy herd of the Institute at Muktesar. They were receiving the following ration:

Five lb. of concentrate mixture consisting of wheat-bran, grain, maize, oil cake and cotton-seed meal for cows giving 8 lb. of milk a day. The grain ration was increased or decreased by ½ lb., subject to a minimum feed of 3 lb., for each pound of milk above or below this quantity. As roughage, the animals received daily 14 lb. of hay per head. In addition they were at pasture for 6 hr. a day when the weather was fine, while during rainy weather the animals were given 28 lb. of green fodder per head.

The carotene content of the fodders, estimated by the method of Ferguson & Bishop(8) was found to be as follows (in mg./100 g.): Concentrate mixture 0·306, hay 6·42, green fodder 25·7 (dry basis). An animal giving 8 lb. of milk was therefore receiving from 400 to 1200 mg. of carotene per day.

Determination of vitamin A in milk

The method was that adopted by Olson *et al.*(9). The final ethereal solution was concentrated under reduced pressure to a known volume (generally 20 ml.) and the intensity of the yellow colour was measured in a Lovibond tintometer. The yellow colour of the solution was assumed to be due entirely to carotene and the total value of carotene was calculated in Moore's yellow units (Moore(10)).

The solution was next evaporated to dryness and the residue dissolved in a small quantity, usually 2 ml., of anhydrous chloroform. To 0·5 ml. of this solution, 2 ml. of saturated antimony trichloride in chloroform were added and the blue colour measured and expressed as Moore's blue units.

The following example will illustrate the efficiency of the method. One hundred ml. of milk were taken from a pooled sample and the remainder was churned into butter after the butter-fat content of the milk had been estimated. The carotene and vitamin A content of the milk was determined according to our method, and similar determinations were done on the butter-fat according to the method of Booth *et al.*(11). The results obtained are shown in Table 1.

Table 1. *Carotene and vitamin A content in whole milk and in the butter-fat separated from the same milk sample*

Sample	Carotene (yellow units)	Vitamin A (blue units)
100 ml. milk	24·4	70·6
1 g. butter-fat	3·6	12·4

Since the sample of milk had a fat content of 5 %, the total amount of carotene and vitamin A in 100 ml. milk calculated on the basis of fat content was 18 yellow units and 62 blue units.

The carotene and vitamin A contents of milk from a number of cows are shown in Table 2.

As found by other workers, therefore, the vitamin A activity of the milk of animals on similar rations is subject to great individual variation. Comparing our figures with ones reported from Europe, it may be pointed out that, whereas the carotene content is generally higher than the corresponding averages for English cattle, the vitamin A value is decidedly lower (Henry *et al.*(12)).

Estimation of vitamin C in milk

The vitamin C in milk was determined by the method of Birch *et al.*(13). To 20 ml. of milk, 5 ml. of 20 % trichloroacetic acid were added; the mixture was then shaken thoroughly and filtered. The filtrate was titrated against a

standard solution of 2:6-dichlorophenol-indophenol and the vitamin C expressed as mg. of ascorbic acid per 100 ml. of milk. Only the reduced ascorbic acid content of the milk was estimated, as it is known that the amount of the reversibly oxidized ascorbic acid present in fresh milk is negligible.

At first, the milk was collected in an ordinary glass flask at 7 a.m. and kept in the laboratory until 10 a.m. when the estimation was carried out. The milk samples thus examined were found to contain only about 0·2 mg./100 ml. of

Table 2. *Carotene and vitamin A content of milk from different breeds of cows*

No. of animal	Breed	Carotene	Vitamin A
1	Hill cow	146	158
2	,,	60	112
3	,,	62	101
4	½ Hol./½ Kar.	60	105
5	,,	90	112
6	½ Har./¼ Hol. ¼ Kar.	106	101
7	,, ,,	44	60
8	½ Hol./¼ Har. ¼ Kar.	78	140
9	,, ,,	24	74
10	½ Hol. ½ Har.	126	132
11	,,	52	90
12	,,	62	131
13	½ Sah./¼ Hol. ¼ Har.	76	174
14	½ Har. ½ Sah.	44	118
15	½ Hol. ½ Har.	52	131
16	½ Hol. ¼ Thar. ¼ Sah.	54	111
Average		71 ±32·03	115·6 ±35·46

Hol. = Holstein; Kar. = Karachi; Har. = Hariana; Sah. = Sahiwal; Thar. = Tharparkar.

Carotene and vitamin A are expressed respectively as Moore's yellow and blue units per 100 ml. milk.

milk. It was then suspected that the ascorbic acid was possibly being destroyed through the action of light (Kon & Watson(7)) and the following experiment proved this to be correct. A sample of milk was collected and its ascorbic acid content determined immediately. Portions of the same samples were placed in two glass flasks, one of which was wrapped in black paper. Within 3 hr. the milk in the uncovered flask had lost most of its ascorbic acid, whereas there had been little or no loss in the sample in the wrapped flask, as may be seen from the following results:

No.	Nature of sample	Ascorbic acid mg./100 ml. milk
1	Analysed immediately	2·19
2	Analysed after 3 hr. (sample kept in a bottle wrapped with black paper)	1·98
3	Analysed after 3 hr. (sample kept in an uncovered flask)	0·19

Owing to the effect of light on the vitamin all future samples were collected in bottles wrapped in thick black paper. The ascorbic acid content of such samples is shown in Table 3.

It will be seen that cow's milk when properly collected and stored does not show such great individual variation in vitamin C content as in vitamin A content. This has also been the experience of other workers, who have shown that the kind of feed has no effect on the vitamin C concentration of milk (Whitnah & Riddel(14); Bartlett *et al.*(6)). It would therefore appear that the low values reported by other Indian workers are probably due to the destruction of the vitamin following storage and exposure to light.

The more constant level of vitamin C in milk, as opposed to the varying levels of carotene and vitamin A, raised the question as to whether the steady vitamin C level is due to the fact that bovines can synthesize this vitamin, whereas their ability to secrete carotene or vitamin A in the milk depends entirely on the intake of these substances in the food. It has been shown by Parsons(15), by Parsons & Hutton(16) and by Lepkovsky & Nelson(17) that rats can be reared successfully for generations on a diet which readily produces

Table 3. *Ascorbic acid concentration of milk*

No. of animal	Ascorbic acid (mg./100 ml.)
3	2·29
5	1·96
6	1·44
7	2·11
8	1·74
9	2·11
10	1·70
11	2·50
12	1·33
13	2·07
14	2·00
15	2·30
16	2·26
Average	1·94 ±0·35

scurvy in guinea-pigs. They presumed that rats do not require any vitamin C in their diet but are able to synthesize it from some unknown constituents of their food. This hypothesis was substantiated by Harris & Ray (unpublished results), who, after breeding rats for two generations on a vitamin C-free diet, observed by the micro-titration method of estimation of vitamin C, as rich a concentration of vitamin C in various tissues as was found in similar tissues of animals on the stock laboratory diet. Apart from rats, dogs (Harris & Innes(18)), pigs (Orr & Crichton(19)), chickens (Hart *et al.*(20)) and sheep (Madsen *et al.*(21)) were also able to synthesize vitamin C.

It appears, however, that few studies have been carried out with bovines on such rigidly controlled diets as those used with rats and guinea-pigs. Thurston *et al.*(22) found that calves thrived well on a ration of autoclaved hay and a concentrate mixture of maize, oats and linseed meal. They presumed, therefore, that bovines do not require vitamin C in quantities large enough to be detected by tests with guinea-pigs. As this experiment was done at a time

when no chemical means of estimating vitamin C were available, it was not possible for these workers to evaluate the total amount of vitamin C, if any, consumed by the calves. This point is of great importance, as it is well known that the requirement of vitamin per kg. of body weight definitely decreases as the size of the experimental animals is increased. Thus a monkey weighing 1 kg. requires the same amount (1 mg.) of vitamin C for its maintenance as is required by a guinea-pig weighing 200 g. A young boy weighing 20 kg. requires for maintenance only 10 mg. of vitamin C. It will therefore be seen that a diet which will furnish only 0·3 mg. of ascorbic acid (vitamin C) to a guinea-pig, and will thus produce scurvy in this animal, will supply a monkey with 3 mg. of ascorbic acid, as the food intake of the monkey is ten times as great as that of guinea-pigs. 3 mg. of ascorbic acid is ample to protect a monkey completely from scorbutic attack.

Examining the diet used by Thurston *et al.*(22), it will be shown in the experimental portion of this article that mere autoclaving does not destroy the vitamin C present in roughage. This fact supports the findings of Weiss(23) that hays may retain sufficient vitamin C to prevent the production of scurvy even when fed to guinea-pigs.

In direct contradiction of the work of Thurston *et al.* is the finding of Bougart (quoted by Hutyra *et al.*(24)). He produced symptoms resembling scurvy in calves fed on boiled milk. Similarly Hjarre & Lilleengen(25) fed very young calves, aged 8–11 days, with a diet of milk poor in vitamin C. The animals were killed after 90 and 131 days and showed lesions of scurvy in their teeth.

In view of these contradictory reports, it was decided to examine the whole question by carrying out a well-controlled experiment. In addition to studying the synthesis of vitamin C by animals, it was also decided to test the truth of Macgregor's(26) theory that vitamin C can be produced in the alimentary canal of farm animals through the germination of seeds contained in the grain fed as concentrates. As it is known that seeds during germination produce abundant vitamin C (Ray(27)) and that the grain mixtures remain in the rumen for 36–50 hr. (Dukes(28)), which may be long enough for germination, it was decided to treat the concentrate before feeding in such a way as to prevent any possibility of germination in the rumen or other part of the alimentary canal.

Experimental

The preparation of a vitamin C-free ration presented unexpected difficulties. The estimation of ascorbic acid (vitamin C) in grain mixtures and in dried fodder was the first obstacle. Extraction of the grain mixture by the usual procedure of grinding with sand and trichloroacetic acid (Birch *et al.*(13)) yielded a highly viscous opalescent solution, while wheat straw gave a deep yellow-coloured solution. It was impossible to titrate either of the solutions against 2:6-dichlorophenol-indophenol solution with any degree of accuracy.

After various trials, the following methods were adopted. 20 g. of grain mixture were ground thoroughly with 20 ml. of 20 % trichloroacetic acid, 1 g. sodium pyrophosphate and a little sand, and the whole mass was mixed with 60 ml. of water to give a final acidity of about 5 % trichloroacetic acid. The pyrophosphate was added to prevent the oxidation of ascorbic acid on standing (Giri(29)). After standing for half an hour, the whole mass was centrifuged and the opalescent supernatant solution separated. Assuming that all the ascorbic acid was present in the aqueous phase and neglecting the moisture content of the grain mixture, 1 ml. of the solution was equivalent to 0·25 g. of the grain mixture. The opalescent solution was mixed with an equal volume of absolute alcohol, mixed thoroughly and centrifuged again. The very slightly opalescent solution could then be easily titrated with a dilute standard solution of 2:6-dichlorophenol-indophenol (1 ml. of dye solution = 0·050 mg. ascorbic acid). The dye solution was added from a microburette to 10 ml. of the alcoholic solution and the amount of dye required to give the first trace of pink colour was noted. A blank was run out at the same time on a dilute alcoholic solution of trichloroacetic acid and the amount of dye required for the blank was subtracted from the original reading. The vitamin C was calculated directly, each ml. of the filtrate representing 0·125 g. of the grain mixture.

For the straw extracts a different method was adopted. It was found difficult to grind the straw with sand and trichloroacetic acid. The straw was therefore heated for an hour on the water bath in an atmosphere of carbon dioxide, with a minimum quantity of 10 % acetic acid solution containing a little sodium pyrophosphate. The flask was then cooled in ice and the mass ground with a little sand. The mixture was centrifuged in large wide-mouthed tubes. A known volume of the dark yellow-coloured supernatant liquid was treated drop by drop with basic lead acetate until precipitation was complete. The solution was filtered at once and the light yellow-coloured solution was titrated as before with dilute standard dichlorophenol-indophenol solution. Amounts equal to 85–90 % of the added ascorbic acid could be estimated, about 10–15 % being lost during precipitation by lead acetate. For this reason, the observed values were increased by 15 % to make up the loss during the clarification treatments.

The calf mixture ordinarily used in this Institute has the following composition:

Wheat bran	...	...	600 parts.
Barley ...	...	...	600 ,,
Maize ...	...	...	300 ,,
Rape cake	...	...	300 ,,
Common salt	...	...	300 ,,

The mixture is almost of the same composition as that used by Thurston *et al.*(22a). Such a mixture was found to contain ascorbic acid in amounts of 1·65 mg./100 g. mixture. The wheat straw available here had a content of

0·85 mg. of ascorbic acid per 100 g. Assuming an intake of 1½ lb. of grain mixture to 3 lb. of wheat straw by a calf of 150 lb. body weight, the total daily intake of ascorbic acid will be about 22 mg. From past experience, it is known that such a diet when fed to guinea-pigs is sure to produce scurvy. Thus diets, which would have been called vitamin C-free from their effect on guinea-pigs contain in reality appreciable quantities of ascorbic acid.

In order to destroy the vitamin present, the concentrate and roughage mixtures were made alkaline by moistening with caustic soda solution and heated in the autoclave at 120° C. for 2 hr. After autoclaving, the values of ascorbic acid were found to be practically unchanged. The autoclaved mass was kept overnight and next morning dried in a hot-air oven at 120° C. and then crushed in a mill. The dried products gave the following figures (mg./100 g.): grain mixture 0·45 and wheat straw 0·28. It was not possible to reduce the figures further. It appears that the residual reducing substances are different from ascorbic acid, which is known to be heat labile.

Table 4. *Concentration of ascorbic acid in the blood plasma of animals fed heated and unheated rations*

	Heated ration		Unheated ration	
Date	B.C. 58	H.C. 55	B.C. 43	H.C. 62
15. xi. 39	0·40	0·47	0·34	0·47
5. xii. 39	0·42	0·56	0·51	0·56
19. xii. 39	0·66	0·78	0·66	0·55
11. i. 40	0·42	0·59	0·58	0·55
14. ii. 40	0·54	0·38	0·61	0·53
25. iii. 40	0·48	0·48	0·70	0·48
26. iv. 40	—	0·46	0·61	0·50
20. v. 40	—	0·51	0·58	0·56
16. vi. 40	—	0·41	0·56	0·46
10. vii. 40	—	0·63	0·60	0·57

B.C. = Bull calf; H.C. = Heifer calf.

Figures show mg. ascorbic acid per 100 ml. blood plasma.

Two hill calves, 6 months of age, were fed on the treated concentrate mixture and wheat straw. Two other calves of the same age were kept as controls and given the untreated calf-mixture and straw. In addition, each animal was given a daily dose of 2 oz. of cod-liver oil, in order to supply sufficient vitamin A. The concentration of ascorbic acid in the blood was examined at intervals and the results are given in Table 4.

It was thought that any signs of subclinical scurvy would be detected if there was a persistent and gradual fall in the vitamin C level in the blood, as from results obtained in humans it is known that a steady fall in the concentration of the vitamin in the blood is the first sign of hypovitaminosis. It will, however, be seen that the concentration of vitamin C in the blood of the animals in the two groups remained more or less constant during the whole experimental period of 9 months. In humans a concentration of 0·4 mg. or above per 100 ml. of blood plasma is taken to be the normal figure. As it has

been shown that the vitamin C concentration in the tissues of normal individuals of various species is more or less the same (Bessey & King(30)) the concentrations given in Table 1 show that the calves were getting sufficient vitamin C to keep up the normal level of the vitamin in the blood. As, by autoclaving and further heating, any embryo plant tissue present in the concentrate mixture must have been destroyed, the possibility of vitamin C being produced by germination of such plants is eliminated.

The only other alternative, apart from that of actual synthesis of the vitamin by the animal itself, is the production of the vitamin in the rumen or in some other part of the alimentary canal by the micro-flora present in these sites. In order to test this calf B.C. 58, one of the animals getting the treated ration, was killed after an experimental period of nearly 6 months. On autopsy, the animal was found to be quite normal and the carcass in good condition.

A few tissues were analysed for their vitamin C content and the following figures (mg./g. tissue) were obtained: liver 0·34, spleen 0·35, kidney 0·18, suprarenal glands 2·10, pituitary gland 0·61. These figures agree reasonably with those of normal animals as found by Birch *et al.*(13), Harris & Ray(31), Bessey & King(30), Svirbely(32) and others. It is apparent therefore that the animal was producing or getting sufficient vitamin C to keep the concentration of the vitamin in the various tissues at the normal level.

Samples were taken of the contents of the rumen, abomasum, duodenum and colon and bacteriologically examined, with the result that from each site five or six different types of bacteria were isolated.

Each bacterial type was inoculated into media prepared from the contents of the organ from which it was isolated, i.e. rumen bacteria were cultured in rumen contents sterilized by autoclaving at 120° C. and so on. Good growths were obtained in nearly all the media after incubation for 36 hr., but either very little or no vitamin C was found to be present. The experiment was repeated by taking, as before, culture media composed of the contents of the various organs, but adding mannose to each tube of medium. Mannose was added because, according to several workers, this carbohydrate can act as a precursor of vitamin C in the animal system (Guha & Ghosh(33), Rudra(34)). The results of this experiment were also negative, scarcely any vitamin being produced.

These experiments tend to support the view that the animal itself can synthesize vitamin C within its body and so is not dependent on any external source. The site of synthesis, as well as the ingredients from which it is produced, are still unknown, but in view of the fact that the vitamin is found in such large concentrations in so many glands and tissues, it is probable that no single tissue or gland is responsible for its production.

Summary

1. A simple method is described for the determination of carotene and vitamin A in milk. Figures for the carotene content of milk were higher and those for vitamin A were lower than similar figures reported by Western workers.

2. Figures for vitamin C in milk are similar to corresponding figures of English and American workers. Previous low values for vitamin C, reported by Indian workers, are due to destruction of the vitamin by light.

3. The vitamin C concentration of milk is not subject to great individual variation, probably because cows cannot excrete in milk any vitamin C taken in with the food and because vitamin C of milk is produced by synthesis within the mammary glands from some simple blood constituents.

4. To test the ability of bovines to synthesize vitamin C, young calves were kept for long periods on a strictly vitamin C-free diet. The concentration of vitamin in the blood and other tissues of these animals remained, however, at a normal level. Calves appear therefore to be able to synthesize sufficient vitamin C for their normal growth and activity.

5. Unsuccessful attempts were made to produce vitamin C in vitro by growing bacteria isolated from various parts of the alimentary canal on media prepared from ingesta taken from the regions from which they were isolated.

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278. STUDIES ON ASS'S MILK

COMPOSITION

By C. P. ANANTAKRISHNAN
Department of Biochemistry, Indian Institute of Science, Bangalore

The chemistry of cow's milk has attracted much attention because of its importance as a commercial food, but knowledge of the chemistry of the milk of other domestic animals is not so wide. The composition of goat's milk under English conditions has been investigated by Knowles & Watkin(1). Gamble *et al.*(2) have carried out a comprehensive study of the protein and fat of goat's milk. Linton(3) has investigated the variations in the composition of mare's milk.

Richmond(4) was one of the earliest workers to investigate the composition of ass's milk. Table 1 gives the composition of such milk, as reported by various workers.

Table 1. *Percentage composition of ass's milk*

Investigator	Water	Fat	Lactose	Casein	Albumin	Ash
Schlossman(5)	88·8	0·36	4·94	0·98	1·33	0·31
Koenig	90·12	1·37	6·19	0·79	1·06	0·47
Richmond(4)	88·9	1·18	6·86	1·74		0·45

Cimmino(6) reported the ascorbic acid content of ass's milk as 6·0–8·5 mg./100 ml. Ribadeau-Dumas & Fonet(7) advised the use of ass's milk as an easily assimilable food, particularly in certain pathological conditions. Bhagvat & Sreenivasaya(8) have shown, by in vitro enzymic digestibility of cow's and ass's milk, the greater ease with which trypsin breaks down the ass's milk proteins. But apart from the above, practically no systematic work appears to have been done on ass's milk.

In many parts of India, ass's milk has long been recognized as a suitable infant food, especially in cases where cow's milk is not tolerated. It is also claimed to possess certain curative properties. In view of these claims and of its close resemblance in composition to human milk, a systematic investigation of ass's milk was undertaken.

Nitrogen distribution in ass's milk

(*a*) *Proteins*

Davies(9) and Knowles & Watkin(1) in their investigations of the nitrogen distribution of cow's and goat's milk respectively, used Moir's method(10) for the protein separation. This method has been used in the present investigation.

The caseins of all milks are the same as regards precipitation properties, although the casein aggregate may be made up of different amounts of the various casein fractions. Whittier (11), working on the buffer intensities of milk, states that casein is the chief factor concerned in the buffer action of milk at *p*H 4·5–4·7. Owing to the different buffering powers of various milks, a different amount of sodium acetate solution has to be added to milk acidified with the standard amount of acetic acid to attain a *p*H of 4·6. This is especially so in watery low protein milks such as those of the human, the mare and the ass.

Trial experiments were carried out on ass's milk to determine the amount of acetic acid-sodium acetate mixture to be added in order to reach a *p*H of 4·6. A series of experiments was then carried out over a *p*H range of 4·3–4·8, to determine the point of maximum precipitation of casein.

Table 2. *The effect of p*H *on the precipitation of casein in ass's milk*

No.	0·25*N* sodium acetate added ml.	*p*H of the filtrate	Casein N per 100 ml. of milk mg.
	Sample A		
I	4·5	4·34	96·4
II	6·0	4·50	109·6
III	7·5	4·56	110·4
IV	8·0	4·58	110·5
V	8·5	4·61	111·4
VI	11·0	4·70	109·1
VII	13·0	4·81	97·8
	Sample B		
I	4·5	4·35	95·6
II	6·0	4·52	97·8
III	7·5	4·59	99·5
IV	8·0	4·60	99·6
V	8·5	4·63	97·4

To 10 ml. portions of ass's milk, diluted to 50 ml. with water at 40°, were added 1·5 ml. of 1·67*N* acetic acid and the liquid was stirred gently. After 20 min., varying amounts of 0·25*N* sodium acetate solution (4·5 ml. or above) were added to the contents of each beaker. After an hour, the contents of each were filtered through no. 42 Whatman filter and the nitrogen in the precipitate was determined by the Kjeldahl method. The *p*H of the filtrates was also determined (glass electrode). The results on two samples A and B, obtained from two different animals, are given in Table 2.

Samples from different animals, and the bulked sample, were also treated in the above manner using 1·5 ml. of 10 % acetic acid and 8·0 ml. of 0·25*N* sodium acetate. The *p*H of the filtrates lay between 4·56 and 4·65.

The amount of sodium acetate solution to be added to 10 ml. of ass's milk acidified with 1·5 ml. of 10 % acetic acid, in order to attain a *p*H of 4·6 ± 0·05, is thus 8·0 ± 0·5 ml.

Globulin and total protein were determined by Moir's method. The albumin nitrogen was calculated as the difference between the total protein

nitrogen, and the casein plus globulin nitrogen. The nitrogen distribution of typical samples are given in Table 3.

(b) The non-protein nitrogen

Milk contains only small quantities of the non-protein nitrogenous constituents, e.g. amino acids, urea, creatine, creatinine, uric acid and traces of other substances. Of these, urea accounts for most of the nitrogen.

For the separation of proteins in this aspect of the work, trichloroacetic acid is not recommended(12). Erickson *et al.*(13) have examined various methods of protein separation and recommended tungstic acid, which has been adopted in the present work.

Table 3. *Nitrogen distributions (mg./100 g.) in milk samples from three animals**

Sample from animal no.	Total N	Total protein N	Casein N	Albumin N	Globulin N
1	281·0	219·0 (80·0)	108·0 (38·4)	117·6 (37·5)	6·0 (2·2)
2	251·6	207·2 (80·8)	107·8 (42·0)	99·4 (35·2)	9·0 (3·5)
3	283·4	222·3 (78·4)	97·6 (34·4)	—	—
4	282·0	217·5 (77·2)	109·8 (38·9)	97·8 (34·8)	9·9 (3·5)
2	276·4	227·6 (78·1)	139·4 (50·0)	67·3 (24·5)	8·9 (3·2)
3	269·0	213·6 (82·0)	107·6 (40·0)	98·3 (37·6)	7·7 (2·9)
1	277·0	220·0 (80·1)	108·1 (39·4)	106·6 (38·5)	7·3 (2·6)
2	283·0	230·1 (81·3)	109·2 (38·6)	112·5 (39·7)	8·4 (3·0)
3	266·0	211·5 (79·1)	98·2 (36·9)	94·8 (36·4)	8·1 (3·2)

Brackets refer to nitrogen expressed as percentages of total nitrogen.

* Animals 1 and 2 were in the third month of lactation, animal 3 in the fourth month.

Table 4. *Non-protein nitrogenous constituents of ass's milk (mg./100 ml.)*

Sample no.	Amino N	Urea N	Uric acid
1	7·5	24·0	0·8
2	7·0	23·7	0·9
3	8·9	23·0	0·9
4	9·6	24·2	0·8
5	6·8	27·7	0·7
6	8·7	23·2	0·2

Preparation of tungstic acid filtrate. Twenty ml. of the ass's milk sample were diluted with 140 ml. of water and 16 ml. each of 10 % sodium tungstate solution and 0·67 *N* sulphuric acid were added. The mixture was made up to 200 ml., filtered, and total nitrogen determined on 10 ml. aliquots of the filtrate.

The following determinations were made on the tungstic acid filtrate: *Amino nitrogen* on 5 ml. aliquots by Van Slyke's gasometric method procedure; *Urea nitrogen* on 5 ml. aliquots by Van Slyke's gasometric method, by the hypobromite reaction(14); *Uric acid* on 5 ml. aliquots by Benedict's direct colorimetric method(15,16).

The results of the determinations of the various non-protein nitrogenous constituents are given in Table 4.

Creatinine and creatine could not be detected in the tungstic acid filtrate.

The results for ass's milk compared with those for cow's and for human milk are given in Table 5. This table shows that ass's milk is essentially similar to human milk in composition.

The ratios of the different forms of nitrogen are given in Table 6. This again shows the similarity of ass's to human milk.

It may be noted that the non-protein nitrogen in ass's milk amounts to about 20 % of the total nitrogen, while the corresponding figure for cow's milk is 6·0 %. Human milk contains about 24·0 % of non-protein nitrogen.

Table 5. *Nitrogen distribution of normal milk*

	Cow (9)	Human (17)	Ass
	*Protein (g./100 g.)		
Total protein	2·96	1·25	1·31
Casein	2·41	0·50	0·70
Albumin	0·38	0·75	0·61
Globulin	0·17	—	0·07
	Non-protein nitrogen (mg. N/100 ml.)		
Total non-protein	25·2 (18)	37·7 (19)	41·0
Urea	9·5	17·9	24·3
Amino	5·4	6·3	8·1
Uric acid	1·6	2·1	0·7

* Expressed as N × 6·38.

Table 6. *Ratios of the different forms of nitrogen in normal milk*

	Cow	Human	Ass
Casein N:albumin N	6:1	1:1·5	1:1
Casein N:N.P.N.	12·9:1	—	2:1
Albumin N:N.P.N.	2·1:1	2:1	1·8:1

The chloride, lactose, and the 'chloride-lactose index' of ass's milk

(*a*) *Chloride content*

The amount of chloride in milk has assumed considerable importance, since it has been shown that an appreciable increase in chloride above the normal indicates a disturbance in milk secretions.

An extensive investigation of the chloride content of cow's milk has been made by Davies (20); Knowles & Watkin (1) and Linton (3) have determined the chloride content of goat's and mare's milk respectively. In the present work, the method of Davies (21) was adopted to determine the chloride content of ass's milk. The results are given in Table 7.

(*b*) *The lactose content*

The A.O.A.C. gravimetric method was adopted in this work for the determination of lactose. The results are given in Table 7, along with the chloride-lactose numbers, i.e. chloride % ÷ lactose % × 100.

The chloride content of ass's milk has not been previously reported. The mean value is 0·037 %, while the values reported for normal human (22) and mare's milk (3) are 0·038 and 0·040 %, and for cow's and goat's milk 0·11 and 0·15 % respectively. In this respect, therefore, ass's milk again resembles human milk.

The 'simplified molecular constant' of Matthieu & Ferré (23) for cow's milk (% lactose + 19·6 × % Cl = 7) applies approximately to ass's milk.

Cow's milk has a 'chloride-lactose index' of 2 to 3·5. Knowles & Watkin and Linton have recorded 3·8 and 0·12–0·15 for normal goat's and mare's milk respectively. For ass's milk the ratio is less than unity. Moruzzi & Tanzi (24) have recorded the ratio for human milk as less than unity.

Table 7. *Chloride, lactose and the 'chloride-lactose index' of ass's milk*

Sample	Chloride mg. %	Lactose %	'Chloride-lactose index'
E	42	6·23	0·68
F	35	6·06	0·58
G	36	6·20	0·58
E	48	6·50	0·74
F	34	6·23	0·55
G	36	5·98	0·60
E	39	5·85	0·67
F	32	6·00	0·53
G	32	6·24	0·52

THE CALCIUM, PHOSPHORUS AND SULPHUR CONTENTS OF ASS'S MILK

In view of the importance of the inorganic constituents of milk in nutrition, a study of the contents and distribution of calcium, phosphorus and sulphur in ass's milk was undertaken.

Calcium was determined as oxalate in carefully ashed milk. The percentages of total solids, solids-not-fat, fat, ash and calcium (as CaO) are given in Table 8, which also summarizes some of the data given above. The average ash content in ass's milk is 0·40 %, while in cow's and mare's milk it is 0·70 and 0·34 % respectively. The average calcium content of ass's milk is 0·08 %, while in cow's milk it is 0·12 %.

Colloidal calcium

It is known that the colloidal calcium phosphate of milk is precipitated along with the calcium caseinate by the action of rennet. Based on this, Ling (25) developed a method for the determination of colloidal calcium in cow's milk which was applied in the present work for the determination of colloidal calcium in ass's milk.

Ass's and human milks do not curdle well with rennet. In some samples of ass's milk, there is a coagulation of casein, but the coagulum consists of fine flakes and not of a good clot as with cow's milk. In a few samples of ass's milk, coagulation does not occur unless calcium chloride solution is added. The experiments described below were carried out on samples which did form a natural clot with rennet.

Fifty ml. of milk were treated with 0·8 ml. of rennet extract at 30°. After standing for an hour the clotted milk was filtered through filter paper. 10 ml. each of the milk and whey samples in 25 ml. measuring flasks were treated with 10 ml. of 20 % trichloroacetic acid and made up to the mark. They were filtered and neutralized after 10 min. and aliquots of 10 ml. of the filtrate were taken for the determination of calcium. The colloidal calcium in milk was calculated as the difference between the total calcium in milk and the total calcium in whey. The results are given in Table 9.

Table 8. *Percentage composition of ass's milk*

Sample	Total solids	Solids-not-fat	Fat	Lactose	Ash	CaO in milk	CaO in ash
E	8·47	7·79	0·68	6·23	0·40	0·11	28·3
F	8·91	8·28	0·63	6·06	0·37	0·11	29·9
G	8·42	7·83	0·59	6·20	0·38	0·10	27·3
E	8·51	7·80	0·71	6·50	0·47	0·12	25·1
F	8·46	7·82	0·64	6·23	0·38	0·10	27·2
G	9·10	8·50	0·60	5·98	0·40	0·12	29·6
E	7·80	7·14	0·66	5·85	0·42	0·13	30·5
F	8·46	7·92	0·54	6·00	0·37	0·12	31·8
G	8·75	8·12	0·63	6·24	0·40	0·11	27·6

Sample	P_2O_5 in milk	P_2O_5 in ash	Ratio $CaO:P_2O_5$	Chloride in milk	Chloride in ash	Chloride-lactose index
E	0·11	28·7	1·02	0·042	10·5	0·68
F	0·12	32·3	1·08	0·035	9·5	0·58
G	0·10	26·5	0·97	0·036	9·4	0·58
E	0·13	27·0	1·06	0·048	10·2	0·74
F	0·12	30·1	1·10	0·034	8·9	0·55
G	0·12	28·6	0·97	0·036	8·9	0·60
E	0·12	28·8	0·95	0·039	9·3	0·67
F	0·12	32·0	1·01	0·032	8·6	0·53
G	0·11	28·8	1·05	0·032	8·1	0·52

Table 9. *Total and colloidal calcium in ass's milk* (*mg.* Ca/100 *ml.*)

Sample no.	Total calcium	Whey calcium	Colloidal calcium
1	85·3	42·8	42·5
2	81·5	44·9	36·6
3	77·3	41·2	36·1
4	80·1	39·6	40·5

The above table indicates that the colloidal calcium in ass's milk accounts for nearly 50 % of the total calcium. In cow's milk the colloidal calcium represents about 67 %.

Phosphorus distribution

Numerous workers have investigated the various forms of phosphorus compounds in milk. Generally, a separation of phosphorus in milk is effected by the use of trichloroacetic acid. In 1926 Lenstrup(26) investigated the phosphorus distribution of cow's and human milk using picric acid as the protein precipitant. Four fractions were obtained, picric acid insoluble protein and lipid phosphorus; picric acid soluble organic and inorganic phosphorus. The next important work on the phosphorus distribution was under-

taken by Graham & Kay (27). Kay, using trichloroacetic acid, observed a slow hydrolysis of lipids in the protein-lipid precipitate and suggested that, if trichloroacetic acid is employed for deproteinization, the precipitate should not remain in contact with the acid for more than a few minutes. Acharya & Devadatta (28) investigated the phosphorus distribution of buffalo milk using the methods of Eggleton & Eggleton (29) with minor modifications. In the present investigation, Graham & Kay's method was used with the following modifications: (1) The deproteinization was carried out under ice-cold conditions to avoid hydrolysis of phosphoric esters. (2) The trichloroacetic acid soluble phosphorus was fractionated into barium insoluble and soluble fractions. The phosphorus was determined colorimetrically (30).

Total phosphorus. Five ml. of the ass's milk sample were diluted to 25 ml., and aliquots of 2 ml., in triplicate, were used for the determination of total phosphorus.

Deproteinization. Twenty ml. of milk were cooled in an ice-bath in 100 ml. measuring flask, and ice-cold 10 % solution of trichloroacetic acid was added to the mark. After 10 min. the mixture was filtered. This filtrate was used for the subsequent determinations.

Aliquots of 2 and 5 ml. were used for the determination of total and inorganic phosphorus respectively.

Barium precipitation. Forty ml. of the trichloroacetic acid filtrate were pipetted into a 100 ml. centrifuge tube containing 2 ml. of 25 % barium acetate solution and subsequently neutralized to phenolphthalein with cold sodium hydroxide. The mixture was kept in ice for 20 min. and then centrifuged. The precipitate was washed twice with 8 ml. of 1 % barium acetate solution, dissolved in a drop or two of normal hydrochloric acid, and diluted to 6 ml. with water. Barium was removed by sodium sulphate and the solution finally made up to 15 ml. This solution for the purpose of convenience will be referred to as fraction *A*. The centrifugate and the washings from the barium precipitate were made up to 75 ml., the barium was removed from 40 ml., and the resulting solution made up to 50 ml. This solution will be referred to as fraction *B*. Total barium precipitable phosphorus and inorganic phosphorus were determined on 1 ml. and 2 ml. aliquots respectively of the fraction *A*. Total barium soluble phosphorus was determined on 5 ml. aliquots of fraction *B*.

Easily hydrolysable ester-phosphorus. Five ml. aliquots of each of fraction *B* and the trichloroacetic acid filtrate were brought to normal with hydrochloric acid and kept in a boiling water bath for 7 min. The solutions were cooled, neutralized and the inorganic phosphorus determined.

For the determination of lipoid phosphorus the method used was that of Graham & Kay.

Phosphorus values obtained by difference. The 'easily hydrolysable' ester phosphorus is calculated as the difference between the value for phosphorus after 7 min. hydrolysis and the value for inorganic phosphorus in the same.

The 'resistant form' of phosphorus is calculated as the difference between the total acid-soluble phosphorus and the value for phosphorus after 7 min. hydrolysis. The results are given in Table 10.

Colloidal inorganic phosphorus. The colloidal inorganic phosphorus is calculated as the difference between the percentage of total inorganic phosphorus in the milk and that of the whey, since during the rennet coagulation, the colloidal form of phosphorus is co-precipitated with casein(25). Whey was prepared as described above. 5 ml. each of whey and milk samples were

Table 10. *Distribution of phosphorus (mg./100 ml.)*

Sample no.	Total P	Total acid-soluble P	Inorganic P in trichloro-acetic acid filtrate	Barium precipit-able in-organic P	Barium soluble P	Resistant P (by difference)	Hydrolys-able P in trichloro-acetic acid filtrate	Barium soluble hydrolys-able P	Lipin P
1	57·75	50·00	30·75	28·2	22·30	5·50	13·75	—	5·22
2	55·20	45·30	26·62	27·00	18·75	5·30	13·38	—	7·55
3	58·75	50·00	25·75	24·00	25·13	7·55	16·70	17·04	5·58
4	60·20	51·25	28·50	28·50	22·70	3·75	18·00	17·63	8·00
5	58·75	50·00	26·62	24·50	24·35	5·50	17·90	—	6·30
6	55·20	46·56	24·25	24·00	19·31	7·56	14·75	16·22	5·93
7	58·80	50·30	28·10	26·20	22·70	3·35	18·55	18·41	4·17
8	58·34	50·00	25·81	24·45	24·31	5·97	18·23	—	6·30
9	61·50	50·00	28·50	26·31	22·00	7·50	14·00	—	5·53
10	64·50	51·80	25·83	24·00	25·10	—	—	—	5·90
Mean	58·80	49·52	27·07	26·00	22·67	5·78	16·14	—	6·05

Table 11. *Colloidal inorganic phosphorus (mg. %)*

Sample no.	Inorganic P in milk	Inorganic P in whey	Colloidal inorganic P (by difference)
1	24·3	9·8	14·5
2	27·8	13·5	14·3
3	26·6	14·8	11·8
4	27·8	12·9	14·9
5	26·3	11·8	14·5
6	27·7	13·9	13·8
7	24·9	14·2	10·7
8	26·4	12·8	13·6
9	26·0	12·0	14·0
10	26·6	12·9	13·7

weighed into 25 ml. graduated flasks and made up to mark with 10 % trichloroacetic acid. After 30 min. they were filtered and 2·5 ml. of the milk filtrates and 5 ml. of the whey filtrates were used for the determination of inorganic phosphorus. The results are presented in Table 11.

The total phosphorus in ass's milk is 59·0 mg., whereas the total phosphorus of cow's milk amounts to 100 mg./100 ml. Human milk is poor in phosphorus (15 mg./100 ml.). From 82 to 88 % of the total phosphorus in human, cow's and ass's milks is acid-soluble phosphorus. Ester phosphorus occurs to the extent of 12 % of the total phosphorus in cow's milk, while 46 and 39 % of the total phosphorus are the corresponding values for the human and ass's milks respectively. In this respect ass's milk is again similar

to human milk. Ass's and human milks contain 46 and 36 % respectively of total phosphorus in the inorganic form; cow's milk contains 70 % in this form. In content of inorganic phosphorus, ass's milk resembles human milk.

The inorganic phosphorus in the trichloroacetic acid filtrate is almost wholly precipitated by barium. The barium precipitable hydrolysable and non-hydrolysable forms of phosphorus in buffalo milk amount respectively to 9 and 11 % of total phosphorus. In ass's milk, such forms of phosphorus are not present. The easily hydrolysable ester phosphorus in the trichloroacetic acid filtrate forms 27·4 % of total phosphorus. In ass's milk, all easily hydrolysable ester phosphorus is in the barium-soluble fraction. No difference was observed in the hydrolysable form of phosphorus between the hydrolysis periods of 7 and 100 min. The easily hydrolysable esters have usually been designated pyrophosphate, since it was originally thought to contain only inorganic pyrophosphate and adenyl pryophosphate. It has now been found that this

Table 12. *Distribution of phosphorus in ass's milk*

	Expressed as	
	mg./100 ml.	% of total phosphorus
1. Total phosphorus	58·9	—
2. Total acid soluble phosphorus	49·5	84·0
3. Organic acid soluble phosphorus	22·7	38·5
4. Easily hydrolysable ester phosphorus	16·1	27·4
5. Inorganic phosphorus	27·1	46·0
6. Colloidal inorganic phosphorus	13·5	23·0

Table 13. *Sulphur content of milks* (*mg.*/100 *ml.*)

	Cow	Human	Ass
Masters (31)	29·2	—	—
Revol & Peccard (32)	27–44	8·2–20	—
Author: Sample I	—	—	15·4
Sample II	—	—	16·5

fraction may also contain adenylic acid, and so more recent publications refer to the whole group as 'easily hydrolysable esters'. By contrast, the hexose and triose phosphoric esters, together with phospho-glyceric and phospho-pyruvic acids, become the 'difficultly hydrolysable esters or resistant esters'. The 'resistant ester P' in the trichloroacetic acid filtrate is 10 % of the total phosphorus in ass's milk, while in buffalo milk this amounts to 5 %.

The complete distribution of phosphorus of ass's milk is summarized in Table 12.

Sulphur content

Sulphur was determined on 40 ml. aliquots using the nitric-perchloric acid digestion of Masters (31) and separation as barium sulphate. Table 13 gives the sulphur content of ass's milk; the values for other milks are given for comparison.

The amount of sulphur in ass's milk is lower than in cow's milk, and roughly comparable to that in human milk.

Analytical values of the fat of ass's milk

Milk fats from different species of animals are known to vary considerably in composition. Human milk fat was found by Bosworth (33) to differ in chemical composition from that of cow's milk in that it contained linoleic and palmito-oleic acids and only a small amount of the volatile acids. He also demonstrated the presence in human milk fat of highly unsaturated acids (arachidonic) and saturated acids of greater molecular weight than stearic acid (saponification equivalent 339). In the present work the composition of ass's milk fat was investigated.

Separation of fat. Fat was separated by extracting a litre of ass's milk containing 20 ml. of potassium-hydroxide solution ($d = 1{\cdot}26$) with a mixture of 600 ml. of ether and 100 ml. of alcohol. The aqueous layer was re-extracted with ether and the combined extract washed with water. After drying with anhydrous sodium sulphate, the solvent was removed at a low temperature and the residue used for analysis. The Reichert, Polenske, Kirschner and iodine (Wij's) values were determined. The results are given in Table 14.

Table 14. *General characteristics of the milk fats*

				Ass	
	Human (33)	Cow (34)	Goat (35)	Sample I	Sample II
Colour	—	Light yellow	White cream	Yellow	Yellow
Butyro-refractometer reading at 40°	—	42·5	—	54·5	54·7
Reichert value	2·5	29·6	31·7	9·6	9·5
Polenske value	0·1	1·9	9·2	8·2	7·6
Kirschner value	—	23·7	19·9	5·9	5·5
Saponification number	205·1	253·3	238·1	258·5	261·2
Iodine value	56·2	41·3	28·8	81·4	90·0

The Reichert and Kirschner values for ass's milk fat are low. The Reichert value of human milk fat is 2·5 and in this respect ass's milk fat is similar. The Polenske number is considerably higher in ass's and goat's milk fat as compared with that of the cow. One characteristic of ass's milk fat is its high iodine value, which is higher than the value for the human milk fat.

Summary

The total solids of samples of ass's milk ranged from 7·80 to 9·10, the solids-not-fat from 7·14 to 8·50, and the fat from 0·54 to 0·71 %.

The nitrogen distribution in ass's milk is: casein 39·5, albumin 35·0, globulin 2·7 and non-protein nitrogen 22·8 % of the total nitrogen. Ass's milk contains: casein 0·70, albumin 0·62 and globulin 0·07 %. The total protein content is 1·39 %. Ass's milk is therefore characterized by a low casein, a low globulin and a high albumin content.

The non-protein nitrogen consists of amino nitrogen 8·1, urea nitrogen 24·3 and uric acid 0·7 mg./100 ml. of milk. The urea content is twice that present in cow's milk.

The mean chloride and lactose contents of the milk samples are 0·037 and 6·1 % respectively.

The average calcium and phosphorus content of ass's milk are 0·081 and 0·059 % respectively. Half the calcium is ionic, and half is in colloidal form.

The phosphorus distribution is: total acid soluble 84·0, acid soluble organic 38·5, easily hydrolysable ester 27·4, inorganic 46·0, and colloidal inorganic 23·0 % of the total phosphorus. The ratio of CaO: P_2O_5 is 1:1. 46 % of the total phosphorus is in ester form; this is high when compared with only 12 % in cow's milk; most of the phosphoric ester forms soluble barium salts, which is a distinguishing feature of ass's milk.

The total sulphur content is 15·8 mg./100 ml.

The fat has a penetrating odour and is coloured orange-yellow. It has an iodine value of about 86, which is much higher than that for human milk fat. The Reichert (9·5) and Kirschner values (5·7) are low.

In general, the composition of ass's milk resembles that of human rather than of cow's milk.

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279. THE ESTIMATION OF LACTOSE IN MILK

By A. K. R. McDOWELL
Dairy Research Institute (N.Z.), Palmerston North, New Zealand

THE estimation of lactose in milk by copper reduction methods gives results which are usually somewhat lower than those obtained by the polarimetric method. An adequate explanation of this discrepancy is still lacking. Recent work(1,2,3) has shown that a second sugar, probably glucose, is present in milk, but the proportion is very small and would not account for the differences in the lactose percentages obtained by the two methods. The present paper reports an examination of several of the methods of lactose estimation.

METHODS

(*a*) *Volumetric copper reduction methods*

The direct volumetric copper reduction method of Lane & Eynon(4) may be applied to milk, as they suggest, by adding the milk, diluted 1:10, from a burette to the boiling alkaline copper solution. Carried out in this way the method is rapid and gives concordant results. It may be still further simplified, however, so that dilution of the milk and filling of the burette with each diluted sample is eliminated. The milk under examination (3 or 3·5 ml.) and also 30 ml. of water are added to the measured quantity of alkaline copper solution and the titration is completed by the addition of 0·5 % lactose solution from a burette. For the final accurate titration the 0·5 % lactose solution is run in to within 0·5 ml. of the end-point before boiling is commenced.

Comparison of results for lactose content obtained by direct titration with results obtained after deproteinization. Clarification of milk with dialysed iron(5) gives a filtrate of the same *p*H as the milk and free from the deproteinizing agent, but the dialysed iron does not remove calcium. As it has been shown that calcium salts cause a decrease in the amount of copper reduced(6,7,8) a further treatment of the dialysed iron filtrate with potassium oxalate becomes necessary. Table 1 gives results showing the effect of clarification with dialysed iron and of subsequent decalcification on the lactose estimation by the volumetric copper reduction method. It will be seen that treatment with dialysed iron results in a lower value for lactose content and that this effect is largely eliminated by the decalcification with oxalate.

Farmer(9) modified Lane & Eynon's method to include a clarification of the milk with lead acetate and sodium fluoride which remove both the protein and the calcium. The effect of clarification of milk samples by Farmer's method is shown in Table 2. There was no appreciable difference in the results except

for sample no. 12 from a cow in the early stages of lactation. Evidently errors due to protein and to calcium balance each other and estimations of lactose on unclarified milk by the volumetric copper reduction method give reliable results.

(*b*) *Iodometric and chloramine-T methods*

Lactose estimations were carried out by the iodometric method of Fine (5) and the chloramine-T method of Hinton and Macara (10), using both dialysed iron (Fine) and phosphotungstic acid (Hinton & Macara) as clarifying agents

Table 1. *Lactose percentages by the volumetric copper reduction method on unclarified milk, on milk clarified with dialysed iron, and on milk clarified with dialysed iron and decalcified with potassium oxalate*

No. of milk sample	Lactose (%)*		
	Unclarified milk	Clarified milk	Clarified and decalcified milk
1	4·65	4·46	4·58
2	5·00	4·76	4·95
3	4·77	4·69	4·82
Average	4·81	4·64	4·78

* All lactose percentage figures are in grams of lactose monohydrate per 100 ml. of milk. Corrections for volume of precipitated protein and fat are made in calculating all lactose percentages.

Table 2. *Lactose percentages by the volumetric copper reduction method on unclarified milk and on milk clarified by Farmer's reagent*

No. of milk sample	Lactose (%)	
	Unclarified milk	Clarified milk
4	4·75	4·75
5	4·98	5·05
6	5·24	5·30
7	4·56	4·60
8	4·87	4·95
9	4·84	4·87
10	4·78	4·84
11	4·59	4·55
12	5·27	5·49
13	4·85	4·97
14	4·50	4·47
Average	4·84	4·89

for the milk. Estimations by the two methods were also carried out on filtrates prepared for polarimetric work from zinc hydroxide and cadmium hydroxide treatment of the milk and suitably diluted.

Comparison of results for lactose content obtained by the direct volumetric copper reduction method with results obtained by the iodometric and chloramine-T methods. Tables 3 and 4 show the results using dialysed iron and phosphotungstic acid respectively as deproteinizing agents. In Table 9 are shown the results by the iodometric and chloramine-T methods on the filtrates

prepared for polarimetric work from phosphotungstic acid, cadmium hydroxide and zinc hydroxide.

From these tables it will be seen that the lactose percentages by the chloramine-T estimation after deproteinization with any of the above reagents agree quite well with those by the volumetric copper reduction method. Results by the iodometric method are considerably higher except where clarification by cadmium hydroxide or zinc hydroxide is adopted. Hinton & Macara's claim that the chloramine-T method is superior to the iodometric method in that it gives a minimum oxidation of possible interfering substances is substantiated by these results.

Table 3. *Lactose percentages by the volumetric copper reduction method on unclarified milk and by the iodometric and chloramine-T methods on milk clarified by dialysed iron*

No. of milk sample	Lactose (%)		
	Copper reduction method	Iodometric method	Chloramine-T method
15	5·20	5·49	5·23
16	4·90	5·11	4·91
17	4·93	5·11	4·94
18	5·10	5·30	5·13
19	4·63	4·94	4·77
Average	4·95	5·19	5·00

Table 4. *Lactose percentages by the volumetric copper reduction method on unclarified milk and by the iodometric and chloramine-T methods on milk clarified with phosphotungstic acid*

No. of milk sample	Lactose (%)		
	Copper reduction method	Iodometric method	Chloramine-T method
20	5·30	5·40	5·34
21	4·95	5·05	4·96
22	5·23	5·37	5·26
23	5·08	5·21	5·08
24	4·71	4·89	4·81
Average	5·05	5·18	5·09

Van der Plank (11) observed with plant extracts that the oxidizing action of hypoiodate on substances other than sugars is far stronger than that of copper. This observation offers a means of detecting the presence of interfering substances by a comparison of the results obtained for sugar concentration with the iodine reagents and with the copper reagent respectively. Clarification can only be considered efficient when the iodine estimation is lowered to a value in close agreement with that obtained by copper. If this criterion is applied to the milk filtrates there appear to be interfering substances in both the dialysed iron and phosphotungstic acid filtrates. Evidently these substances are oxidized by hypoiodate and their removal by zinc hydroxide or cadmium hydroxide is essential as a preliminary to the iodometric method.

(*c*) *Ferricyanide reduction methods*

Both Gohr (12) and Blackwood (13) have applied the ferricyanide reduction method of Hagedorn & Jensen (14) for the estimation of reducing sugars to the determination of lactose in milk. Blackwood's technique in the present author's hands did not yield results of sufficient accuracy to warrant its use. An inspection of Blackwood's own figures for standard lactose solutions shows that an error of up to 3 % may be expected.

Gohr's method was more successful, but duplicate results varied too greatly to inspire confidence in the method. Several determinations of lactose in milk by Gohr's method showed higher results in filtrates from clarification of the milk with zinc ferrocyanide (Gohr) than in filtrates from cadmium hydroxide clarification (Blackwood), presumably due to interfering substances in the former filtrate.

(*d*) *Polarimetric methods*

A Hilger 400 mm. polarimeter with Lippich triple field polarizer was used. An electric sodium lamp provided the source of light.

Various protein precipitants for the clarification of milk for polarimetric work are described in the literature and the use of several of these was investigated. The A.O.A.C. acid mercuric nitrate treatment as modified by Garrison (15), the phosphotungstic acid precipitant of Hinton & Macara (10), and a zinc ferrocyanide precipitant (16), all gave clear filtrates, but dialysed iron, which is claimed to be satisfactory for condensed milk (7), never gave a perfectly clear filtrate nor was all the protein matter eliminated.

The use of cadmium hydroxide as a deproteinizing agent has been found very effective both for blood (17) and for plant extracts (18) and is said to eliminate all the 'residual reduction' compounds. Blackwood (13) applied the method to obtain a 1:10 protein-free dilution of milk. Some experimental work showed that cadmium hydroxide could be used to obtain the 1:2 deproteinized dilution of milk required for polarimetric work, giving a beautifully clear filtrate.

Reagent. $3CdSO_4.8H_2O$, 26 g.; 1 *N* sulphuric acid, 127 ml.; distilled water to 150 ml.

Method. To 50 ml. of milk in a 100 ml. flask is added 20 ml. of the reagent with shaking. Then 25 ml. of 1·1 *N* caustic soda is added slowly with continuous shaking, the whole made up to the mark with water, well shaken and filtered.

When much of the work with the cadmium reagent was completed Garrison's adaptation to milk (19) of Somogyi's zinc hydroxide precipitant for blood (20) came to the author's notice. By the addition to 50 ml. of milk of 15 ml. of each of the 15 % zinc sulphate and 0·75 *N* caustic soda solutions and making up to 100 ml. with water, this reagent also appeared to give an excellent filtrate.

With all polarimetric estimations of sugar in milk a correction for volume

of precipitate must enter into the calculation of the lactose percentage. Several methods for determining this correction have been suggested (7, 8, 16, 21, 22) and a satisfactory figure has been obtained for the correction of phosphotungstic acid and sodium ferrocyanide precipitates from condensed milk (16).

The correction is due mainly to the volume of precipitated protein and fat, and from a knowledge of the percentage content of these constituents in the milk and their specific volumes it may be calculated. In addition, a smaller correction due to the volume of precipitated reagent may be necessary. Using Scheibe's (8) method of determining the total correction it was found that for 50 ml. of milk, deproteinized and diluted to 100 ml., this smaller volume

Table 5. *Lactose percentages by the volumetric copper reduction method on unclarified milk and by the polarimetric method on milk clarified with acid mercuric nitrate*

No. of milk sample	Lactose (%)	
	Copper reduction method	Polarimetric method
15	5·20	5·58
16	4·90	5·09
17	4·93	5·21
18	5·10	5·41
19	4·63	4·85
Average	4·95	5·23

Table 6. *Lactose percentages by the volumetric copper reduction method on unclarified milk and by the polarimetric method on milk clarified with phosphotungstic acid*

No. of milk sample	Lactose (%)	
	Copper reduction method	Polarimetric method
20	5·30	5·71
21	4·95	5·21
22	5·23	5·54
23	5·08	5·36
24	4·71	4·92
Average	5·05	5·35

was approximately 0·5 ml. and may be taken as such without introducing an appreciable error into the lactose percentage whatever the reagent used.

Comparison of results for lactose content obtained by the direct volumetric copper reduction method with results obtained by the polarimetric method. Tables 5 and 6 show that polarimetric results for lactose from milk deproteinized with either acid mercuric nitrate or phosphotungstic acid are 0·2–0·4 % higher than by the volumetric copper reduction method. Clarification with zinc ferrocyanide gave very similar results.

In Table 7 lactose percentages by the polarimetric method are compared for three deproteinizing agents—cadmium hydroxide, zinc hydroxide and phosphotungstic acid—and are definitely lower after clarification with the first two reagents than with the phosphotungstic acid.

From Table 9 it will be seen that for samples 12, 13 and 14 these lower lactose percentages agree fairly closely with the percentages obtained by the volumetric copper method on milk clarified with Farmer's reagent.

Determinations of total nitrogen (Table 8) in the filtrates for polarimetric work from samples 25, 26 and 27 expressed as percentages of the total nitrogen in the milk prove that cadmium hydroxide and zinc hydroxide are both more efficient in their removal of nitrogen compounds from the milk than is phosphotungstic acid. Richmond (23) called attention to the fact that proteins and their products of hydrolysis are usually laevorotatory, and both he and Garrison (15) found that addition of phosphotungstic acid to an acid mercuric nitrate filtrate gave a higher polarimetric reading. Evidently the cadmium

Table 7. *Lactose percentages in milk by the polarimetric method using cadmium hydroxide, zinc hydroxide and phosphotungstic acid as deproteinizing agents*

No. of milk sample	Lactose (%)		
	Cadmium hydroxide	Zinc hydroxide	Phosphotungstic acid
25	4·53	4·53	4·66
26	4·75	4·73	4·81
27	4·55	4·58	4·68
28	4·70	4·71	4·82
12	5·55	5·59	5·79
13	5·03	5·02	5·26
14	4·48	4·48	4·66
Average	4·80	4·81	4·95

Table 8. *Total nitrogen in the filtrates from cadmium hydroxide, zinc hydroxide and phosphotungstic acid treatments of milk expressed as percentages of the total nitrogen in milk*

No. of milk sample	Nitrogen (%)		
	Cadmium hydroxide filtrate	Zinc hydroxide filtrate	Phosphotungstic acid filtrate
25	3·9	3·8	4·2
26	3·7	3·6	4·3
27	4·9	4·7	5·5
Average	4·2	4·0	4·7

and the zinc reagents remove in addition some compound or compounds which are strongly dextrorotatory and which may include monoamino acids.

Comparison of results for lactose content obtained by the volumetric copper reduction method, by the iodometric methods and by the polarimetric method. It appeared possible that the greatest differences in lactose percentages estimated by different methods, or by the same method applied to different filtrates from the same milk, might be expected in samples from cows in the first few weeks of lactation. A series of determinations was therefore carried out on three specially selected samples of milk. Samples 12 and 13 were from cows 7–14 days after calving and sample 14 from a cow 3–4 weeks after calving. The results are shown in Table 9.

Where the milks had been clarified with the reagents which had been found most suitable for each method (i.e. excluding the phosphotungstic acid figures) all four methods of estimation of lactose gave results which agreed reasonably well. As the major discrepancies in the percentages by different methods had been eliminated it would appear that these figures indicate the true percentage of milk sugar.

Table 9. *Lactose percentages by the volumetric copper reduction method on unclarified milk and on milk clarified by Farmer's method; by the iodometric and chloramine-T methods on milk clarified with phosphotungstic acid, with cadmium hydroxide and with zinc hydroxide; and by the polarimetric method on milk clarified with phosphotungstic acid, with cadmium hydroxide and with zinc hydroxide*

	Lactose (%)				
	Volumetric copper method		Iodometric method		
No. of milk sample	Unclarified milk	Clarified milk	Phosphotungstic acid	Cadmium hydroxide	Zinc hydroxide
12	5·27	5·49	5·88	5·59	5·55
13	4·85	4·97	5·23	5·04	4·97
14	4·50	4·47	4·62	4·46	4·45
Average	4·87	4·98	5·24	5·03	4·99

	Lactose (%)					
	Chloramine-T method			Polarimetric method		
No. of milk sample	Phospho-tungstic acid	Cadmium hydroxide	Zinc hydroxide	Phospho-tungstic acid	Cadmium hydroxide	Zinc hydroxide
12	5·56	5·49	5·54	5·79	5·55	5·59
13	4·99	5·01	5·00	5·26	5·03	5·02
14	4·52	4·45	4·50	4·66	4·48	4·48
Average	5·02	4·98	5·01	5·24	5·02	5·03

Summary and conclusions

1. The direct volumetric copper reduction method of estimation of lactose in unclarified milk gives results which are in good agreement with those on the same milk clarified and decalcified.

2. For the polarimetric or iodometric methods of estimation of lactose the milk is best clarified with either zinc hydroxide or cadmium hydroxide. Clarification with acid mercuric nitrate or phosphotungstic acid for the polarimetric method and with dialysed iron or phosphotungstic acid for the iodometric method gives high results owing to failure to remove interfering substances.

3. Dialysed iron, phosphotungstic acid, cadmium hydroxide or zinc hydroxide are all suitable for deproteinizing the milk for the chloramine-T method of estimation of lactose.

4. The values for lactose content of milk as estimated by the above methods show good agreement and therefore may be accepted as the true milk sugar content.

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280. THE RELATIONSHIP OF METHODS OF BACTERIOLOGICAL EXAMINATION TO THE ERADICATION AND CONTROL OF MASTITIS (*STREPTOCOCCUS AGALACTIAE*)

I. THE USE OF AN ENRICHMENT TECHNIQUE IN REVEALING STREPTOCOCCAL INFECTIONS OF THE COW'S UDDER

II. *STREPTOCOCCUS AGALACTIAE* INFECTIONS IN HEIFERS

By A. T. R. MATTICK, P. M. F. SHATTOCK and M. MOREIRA JACOB[1]
National Institute for Research in Dairying, University of Reading

It is now accepted that 'contagious' bovine mastitis (*Streptococcus agalactiae*), including the subclinical form, is a major cause of loss to the dairy industry. Several estimates(1,2) of the incidence of mastitis and the money value lost in Great Britain have been made, and these are of such dimensions as to demand serious attack on its spread. The extent of the literature(3,4) is evidence of the world-wide interest in the eradication and control of the disease.

In this country Minett *et al.*(5,6) were the pioneers in attempts at eradication, and after extended observation in several herds they concluded that eradication was possible. The basis of eradication was bacteriological examination of the milk of each quarter of each cow at three-monthly intervals with removal of bad cases and the segregation of infected from non-infected cows. These workers considered the recruitment of the herd from first-calf heifers to be most desirable, as they believed that infection was infrequent in animals of this age. Following Minett *et al.*(5,6) others attempted to eradicate the disease by these methods, but disconcerting breakdowns in herds believed to be free from infection took place. This, together with the apparent impossibility of freeing some herds, shook confidence in this method as a means of actual *eradication*. Bull *et al.*(7) in Australia reported that they were unable to maintain an isolated heifer herd free from the disease, and American opinion is divided on the question of the possibility of easy and complete eradication under field conditions. Hucker(8), in fact, found it impossible to maintain a herd free from cows discharging streptococci. Minett(9) in a recent paper showed that by altering the methods of bacteriological examination, many positive cows could be demonstrated in a herd believed to be free and reversed his opinion that eradication in general is feasible. Minett also redirected attention to the work of Steck(10), who holds it to be impossible, because of what he calls 'cultural latency', to free herds from streptococcal infection.

[1] British Council Scholar, Ministerio da Agricultura, Lisboa.

The work which is reported in this paper arose incidentally from the fact that, for research purposes, it was desired to secure a supply of milk from cows without udder infection or abnormality affecting the composition of the milk.

When Minett's paper(9) appeared the observations already made on several herds were found to confirm his contentions. Shortly after the work was begun deficiencies were observed in the usual bacteriological methods and the scope of the original plan of work had to be widened. A large-scale attempt at eradication[1] of mastitis remained the central objective, but comparisons of the value of several methods of bacteriological examination were made. The incidence of *Str. agalactiae* in heifers in isolation and in first-calf animals generally was also determined. These two aspects of the problem are dealt with in this paper.

It may be useful to review the possible causes of the breakdowns in eradication schemes that have occurred. It has been confidently asserted by some workers, not without some evidence, that the udder is the chief reservoir of *Str. agalactiae* and that infection from cow to cow takes place mainly from this source in the course of milking, e.g. by way of the hands of milkers. But in isolated herds believed after numerous bacteriological examinations to be 'free' and then shown to be infected, it it not essential to postulate infection from cow to cow only. It is as likely that the inadequacy of the experimental bacteriological methods is primarily responsible for failure to detect light infections which later become clinical for other reasons. Eradication schemes undertaken in this country have been carried out mainly by the use of Edwards's technique(11). Edwards's medium contains crystal violet and was devised originally for the examination of samples which had travelled to the laboratory. The medium contains a bacteriostatic substance that inhibits the growth of staphylococci, spore formers and some other organisms. It is therefore probable that a proportion of the *Str. agalactiae* present will also be inhibited and when numbers are small none may appear upon the plates of Edwards's medium. Another factor which may affect an eradication scheme, based upon bacteriological examination, is the interval between the examinations. If the accepted routine method of examination is not really searching it may easily be shown statistically that its success is largely a function of the frequency of examination. In practice it may be very difficult to examine large numbers of cows or herds at frequent intervals by methods of the accuracy necessary to reveal *all* infections, but this does not disturb the argument. It is also possible that when samples, originally containing small numbers of *Str. agalactiae*, have travelled under unfavourable conditions and are then examined, no colonies of this organism will grow, thus giving rise to false negative results. The quantity of milk which is examined and the success of methods aimed at concentrating the organisms present are, of course, also determining factors.

[1] Eradication is taken to mean the formation of a herd in which *Str. agalactiae* cannot be demonstrated bacteriologically.

Although the appearance of positive cases in a herd believed to be negative may be partially explained by the inadequacy of bacteriological methods, the primary source of infection, in many cases, remains to be discovered. Minett (9) in discussing the relevant literature points out that calfhood infection, which remains latent, may account for positive cases amongst first-calf heifers. But if infection is in most cases passed from cow to cow as was believed (but is now also questioned by Bull *et al.* (7)), it would not be surprising that cases of infection in first-calf animals which have joined the herd should be found. It would be evidence of some strength if it could be shown that first-calf heifers were infected whilst still in isolation and *before* joining the herd. On this point evidence is given later.

METHODS OF SAMPLING, ETC.

In investigating the role of bacteriological methods in revealing positive cases in herds under control we compared the results of the examination of portions of each sample by the methods given below.

All the samples examined were fresh (usually about 4 hr. old), except with Herd C, from which the samples were 18 hr. old. All the samples were of fore milk after the rejection of a small quantity to flush the teat duct. In our experience great difficulty may be caused by the growth of organisms not streptococci, if the first few streams are not rejected. Care was taken in cleaning the cows and hypochlorite solution was used to disinfect swabs and hands, to avoid the passage of organisms from one sample to the next.

METHODS OF EXAMINATION OF MILK SAMPLES

Amongst several rapid methods for revealing the presence of *Str. agalactiae* sodium azide broth (12) is outstanding and has been shown to give about the same results as Edwards's plating method. To assess its value in relation to plating and to a modified Hotis test (13) using samples from cows believed to be free from infection or only lightly infected, the following examinations were carried out on each single quarter sample.

(1) Plating half of the centrifuged deposit from 10 ml. of milk on Edwards's aesculin crystal violet (cow) blood agar.

(2) (*a*) Edwards's sodium azide broth technique, exactly as described by the author.

(*b*) Streaks were made on aesculin crystal violet blood agar plates, poured previously, from azide broth tubes which showed deposits or were cloudy.

To prove that the azide broth was capable of supporting the growth of *Str. agalactiae*, a small loopful of a culture was transferred from serum Lemco broth and it was observed that growth was good.

(3) *Enrichment method* (modified Hotis test). 9·5 ml. of milk was added to calibrated tubes containing 0·5 ml. of an alcohol/water solution of brom-cresol-purple to give a final concentration of 0·025 %. The tubes were

incubated for 24 hr. at 37° C. or until it could be seen that no change in reaction was likely to take place. Those tubes which were yellow after 24 hr., were thoroughly shaken and heavy streakings made on aesculin crystal violet blood agar plates which were incubated for 48 hr. at 37° C. Further incubation did not affect the result. Later all tubes were streaked whether they showed changes in appearance or not. Sometimes, as described by Hotis, small yellow aggregates or 'balls' appeared on the sides of the tubes. These, as was also observed by McCulloch & Fuller (14), did not always prove to be *Str. agalactiae*, although in the main they were a useful guide to the presence of streptococci. A very thorough examination of the plates (1, 2*b* and 3) was made under a binocular low-power microscope and all likely and indeed many unlikely colonies were picked into serum Lemco broth, which was preferred to serum dextrose broth, as formation of chains by the streptococci appeared to be better. After incubation at 37° C. for 48 hr. a catalase test (streptococci give a negative result) was carried out and a microscopic examination of each tube made for the presence of streptococci. This permitted the rejection of the culture or its reservation for further study. Cultures were then streaked, purified and their reactions determined in mannitol, salicin, raffinose, arabinose, inulin, sorbitol and aesculin broths. The final *p*H in dextrose broth was determined by the glass electrode and the hippurate test (15,16) was carried out. Finally, the identities of many strains were established by the precipitin test using group B specific sera (17).

PART I. COMPARISON OF DIFFERENT METHODS INCLUDING AN ENRICHMENT TECHNIQUE

Experimental results

The results of the examinations provided data for comparing the efficiency of the methods described for revealing the presence of *Str. agalactiae* in milk.

Herd A. The herd on this farm consisted of pure-bred Shorthorns and Guernseys, was 'Attested' and examined regularly by agglutination for *Brucella abortus* infection from which it was virtually free. For many years examinations for mastitis by various methods had been made and there had been remarkable freedom from clinical cases. Examinations by the Edwards's technique began in February 1938 and continued at irregular intervals until November 1939, when the examinations reported in Table 1 were begun. Any cow whose milk gave a positive result on Edwards's medium was segregated from the rest of the herd.

The results in Table 1 were obtained with quarter samples. It is clear that the sodium azide broth and Edwards's crystal violet aesculin blood agar fail to reveal a large number of positive samples. In a total of 177 examinations 71, or more than seven times as many, were found by the enrichment method as by the azide test (10 positives) and fourteen times as many as by the Edwards's medium (5 positives). It appears therefore that the azide medium,

owing perhaps to the bacteriostatic substance present and to the small volume of cream used, fails when the numbers of streptococci present are very small. In streaks on the surface of Edwards's medium, however, growth is excellent, when numbers have been reinforced by enrichment. It should nevertheless be remembered that it is still possible to fail to return as positive samples in which incubation has not led to considerable increases in the small numbers initially present. We have in fact some evidence that small numbers may fail to proliferate in milk samples on incubation at 37° C. Thus we found that although the presence of streptococci could not be demonstrated in 10 ml. quantities in some milk samples, they were easily demonstrated in dilutions of the same milk in sterile separated milk after incubation. This may tentatively be attributed to bacteriostatic, bactericidal, or other substances or mechanisms, in concentrations sufficient to inhibit growth or even to destroy the organisms. All these tubes contained 0·025 % brom-cresol-purple, and the fact that growth took place in the higher dilutions and not in the undiluted

Table 1. *Results of examinations of quarter samples by various methods*

	Sodium azide broth		Streakings on Edwards's medium from sodium azide broth (col. 1)		Plating on Edwards's medium of centrifuged deposit		Streakings on Edwards's medium from incubated samples (10 ml.) (modified Hotis test)	
Herd	+	−	+	−	+	−	+	−
A	10	167	16	161	5	172	71	106
B	0	83	0	83	3*	80*	39	44
C	0	72	0	72	0	72	9	63
D, E	4	117	4	117	2	119	14	107

+ = *Str. agalactiae* recovered. − = *Str. agalactiae* not recovered.
* = 1 ml. milk used for plating.

milk warrants the assumption that in itself brom-cresol-purple at the strength used is without significant bacteriostatic power even when numbers are small.

Herd A had been tested at irregular, but not infrequent, intervals by the Edwards's technique, yet on simultaneous examination by the methods described large numbers of quarters were actually shown to harbour *Str. agalactiae* (Table 1). It will be appreciated that it was necessary to carry out the laborious picking and identification of colonies in order to anticipate the obvious criticism that the Edwards's technique was not being properly used. However, we believe that when it is asserted that there were no colonies of *Str. agalactiae* on the plates there were none in fact. It was also necessary to observe the greatest care in taking the samples and to ensure that organisms were not transferred from cow to cow.

Although the numbers of infected quarters were found to be so large, it is still possible that they would have been rather larger if dilutions of the milk had also been incubated before streaking.

Little (18) has also used an incubation-enrichment technique but he added brom-cresol-purple plus sodium azide. His findings are therefore subject to

the same criticism as those of other workers, who for good reasons in routine work, have used bacteriostatic substances, viz. that small numbers of streptococci as well as the adventitious organisms may be suppressed. Little's paper is discussed more fully later.

Herd B. Herd B contained some 150 cows in milk and quarter samples had been examined for the presence of *Str. agalactiae* by the direct plating of 1 ml. of undiluted milk on four occasions at intervals of approximately 4 months (19). Positive animals were segregated in the usual way.

The results (Table 1) are striking in that of eighty-three samples none was positive in azide and only three on Edwards's medium, whereas no less than thirty-nine samples were proved to contain *Str. agalactiae* by enrichment and streaking. Moreover, by enrichment *Str. dysgalactiae* was found in fifteen cases and *Str. uberis* in two, whereas on Edwards's medium *Str. dysgalactiae* was found once and *Str. uberis* not at all. There was no evidence of clinical mastitis due to either of these organisms.

Herd C. Herd C (Table 1) was under the care of a veterinary colleague and quarter samples had been examined only 5 days before by Edwards's technique with no positive results. An earlier test had also been completely negative. No samples were positive by this technique in our hands, but nine samples out of seventy-two proved to be positive by enrichment. All samples examined by the azide technique were also negative for *Str. agalactiae*. Two further samples were shown to contain *Str. uberis*, by the enrichment technique.

Herds D and E. Herd D was an 'Attested' herd consisting of pedigree Guernseys and Shorthorns. As the herd was recruited from heifers only 3 years ago, the animals were young. Since its establishment the herd has been self-contained and it is also important to note that no cows had ever been kept on this farm previously. The main interest lies in the fact that the herd had been tested monthly, using the Edwards's technique, for eight consecutive months. Of fifty cows nineteen were found to be positive at the first test and a smaller number, one in each month, in the last 4 months (Table 2). There was then an interval of 2 months when, again by Edwards's technique, none out of fifty-four cows was positive. Simultaneous tests by the enrichment method and by Edwards's method showed, however, that three cows were actually positive. Five months later no cows were positive by either method, although these three animals remained in the herd.

This result is very different from that in Herds A and B, which had been tested at less frequent intervals.

The results in Herd E (Table 2), tested nine times by Edwards's technique in 16 months, are similar, in that of thirty-nine cows (all negative by Edwards's method) only two were positive by the enrichment method at the last test.

These two farms provide evidence that even methods which do not detect all light infections may, if used sufficiently frequently, result in the ultimate detection of a much higher proportion of infected animals than when used infrequently, say at three-monthly intervals.

It is therefore possible that infection, even when very light, may pass from cow to cow, but the contention that light infections even in the so-called 'commensal' form are otherwise acquired in many cases is not disposed of. It seems that a high proportion of cows in ordinary herds may harbour streptococci in small numbers. On injury to the udder or on the occurrence of other abnormality in the cow clinical streptococcus mastitis may ensue.

The experience in these two herds is much the same as has been reported to us privately by colleagues working under field conditions, viz. the difficulty, when using Edwards's medium (which has been shown to fail to reveal a high proportion of lightly infected animals), of freeing a herd from infection especially when examinations are not carried out at short intervals. It must be emphasized that no cow or heifer had been admitted to the herds unless she

Table 2

	Herd D			Herd E		
Month	No. cows tested	No. positive on Edwards's medium	No. positive by modified Hotis test	No. cows tested	No. positive on Edwards's medium	No. positive by modified Hotis test
1939: Oct.	50	19	.	39	10	.
Nov.	50	2	.	33	2	.
Dec.	50	1	.	26	0	.
1940: Jan.	48	3	.	30	0	.
Feb.	50	1	.	.	.	.
Mar.	52	1	.	.	.	.
Apr.	57	1	.	.	.	.
May	61	1	.	37	2	.
June	.	.	.	37	0	.
July	.	.	.	37	0	.
Aug.	54	0	3	.	.	.
Sept.	53	0		.	.	.
Oct.	.	.	.	35	1	1
Nov.	.	.	.	.	.	.
Dec.	48	1	.	.	.	.
1941: Jan.	.	.	.	39	0	2
Feb.	46	0	0	.	.	.

proved negative to Edwards's plating test at 10–20 days after calving. During this time all animals remained in isolation. A single negative test may manifestly be insufficient to guarantee that a heifer joining the herd is not infected. Our records show that of 377 first-calf heifers whose milk was examined 81, or 21 %, were infected (Edwards's medium) and that 17, or 4·5 %, showed positive results whilst still in isolation. It is probable in view of the above results that owing to the proved inadequacy of a single examination some lightly infected animals were in fact allowed to join the herd. The danger of introducing an outside strain of *Str. agalactiae*, perhaps of a different serological type (20) or invasive power, cannot at present be estimated, but it may be very real. Unwitting introduction of infection through heifers which appear to be free may account for the appearance of so-called fresh infections (these may in reality only be 'undiscovered infections') in herds believed to be free.

The case of a nurse cow (she did not suckle calves) at a calf-rearing farm in the same group of farms as Herds D and E is of interest. No other animal in milk was kept on the premises and this cow—a first-calf heifer—came from a 'clean' farm (D) after two consecutive negative tests by Edwards's plating method. After further repeated negative tests the cow was finally proved by the enrichment method to harbour *Str. agalactiae* in the udder after she had been in what amounted to complete isolation for 7 months. Later she proved to be positive by Edwards's method and later still she was again negative to both tests. The milker had no contact whatever with other cows. This case tends to confirm the contention that if tests are sufficiently frequent and the methods sufficiently searching, *Str. agalactiae* is likely to be found in animals which may for long appear to be free from infection. Where the method does not detect small numbers, the period of apparent freedom may be very extended (Table 3). In any case a single examination by any method is not to be relied upon.

Table 3

Cows in a 'clean' herd	Edwards's technique: no. of negative tests prior to positive test
G. 504	11
G. 582	4
G. 671	2
G. 686*	5
G. 687	5
G. 695	3
G. 396	9
G. 446	7
1433	4
1471	3
G. 559	9

* Injury to udder 2 months before becoming positive.

Little (18) has reported the mastitis history of several herds and recommended the use of fore milk, without rejection of the first two or three streams, as is the usual practice here. He then used only 1 ml. of a 1/10 dilution for plating on blood agar without a bacteriostatic substance. In the event of a mild teat duct infection, which he suggests may be the case when numbers are small, it is reasonable to suppose that some organisms will be washed out if the first few streams are rejected. In our opinion, however, the inclusion of sodium azide in the milk before incubation, as in Little's alternative method, together with the fact that only 1/10 ml. was subsequently used for cultivation, make it likely that light infections might have been missed, as even after incubation we have often found numbers on the streaked plates to be very small.

There is moreover considerable danger of failure to detect light infections —whether of the haemolytic or non-haemolytic type of *Str. agalactiae*—if plates are over-crowded with other organisms when blood agar without a bacteriostatic substance is used, even though samples are very carefully taken

from clean cows. In our own work we have preferred to use blood agar with a bacteriostatic substance (Edwards's medium) *after* enrichment of the small numbers originally present in the milk.

Herd F. This herd was recruited entirely from positive animals which had been removed from other herds under examination by Edwards's technique. The numbers of colonies on the plates made from the milk of different cows varied from one to very large numbers and the animals had been in the herd for periods varying from 2 months to nearly 3 years.

It should be emphasized that *Str. agalactiae* had previously been recovered by Edwards's technique from every cow. Although the plates from many cows showed far fewer than twenty colonies, the animals were at once segregated.

These examinations (Table 4), as in the other herds, were made to compare Edwards's with the enrichment method, but the failure to detect all positive cases at the first examination by the enrichment method, e.g. Table 4, cow 455, again emphasizes the necessity for frequent examinations even by a method known to detect small numbers of streptococci. It may perhaps be argued that positive results after repeated negatives, in a herd known to contain positive cows however lightly infected, may be due to recovery and reinfection, but in that case it must be admitted that even small numbers may be dangerous and that to fix an arbitrary number, above which animals are segregated, and below which they are not, is not beyond criticism. The point of special interest in this herd is the persistency of small numbers of streptococci over long periods. It is evidently unsafe to assume that small numbers of streptococci are unlikely to persist.

Frequency of occurrence of streptococci other than Str. agalactiae

On the whole the samples examined proved to be remarkably free from non-pathogenic streptococci. For example, *Str. lactis* and *Str. bovis* were only rarely found, whether by enrichment or by Edwards's method. This is perhaps merely a reflexion of the cleanliness of sampling. On the other hand, by enrichment, in 449 examinations, *Str. dysgalactiae* was isolated on twenty occasions, chiefly from one herd, although it occurred in others. The actual numbers of organisms present again appeared, in many cases, to be very small, and it seems that the streptococci causing the so-called acute types of mastitis may be present in the udder of the cow in a state of latency as with *Str. agalactiae*. Several cases of mastitis following injury have been found to be due to *Str. dysgalactiae* and in one case a heavy infection in a first-calf heifer, still in isolation, was discovered.

In 449 examinations, *Str. uberis* was also found on twenty occasions, in all cases without evidence of clinical symptoms.

Mixed Str. agalactiae *and* Str. dysgalactiae *infections*

Several cases of mixed infection in the same quarter were discovered.

Table 4. *Herd F*

Cow no.	Dates tested*	Result of test	No. of quarters from which *Str. agalactiae* was isolated	No. of *Str. agalactiae* colonies on Edwards's medium
355	12. i. 38	+	N.R.	N.R.
	23. iii. 38	+	1	N.R.
	20. x. 39	−	0	0
	16. xi. 39	−	0	0
	15. xii. 39	−	0	0
	22. v. 40	−	0	0
	5. vi. 40 M.H.	+	1	.
394	24. xi. 37	+	N.R.	N.R.
	12. i. 38	+	N.R.	N.R.
	20. x. 39	−	0	0
	16. xi. 39	+	1	∞
	22. v. 40	+	1	100
	5. vi. 40 M.H.	+	2	.
414	4. x. 38	+	1	300
	20. x. 39	−	0	0
	22. v. 40	−	0	0
	5. vi. 40 M.H.	+	1	.
429	12. i. 38	+	N.R.	N.R.
	23. iii. 38	+	N.R.	N.R.
	20. x. 39	−	0	0
	16. xi. 39	+	4	1, 2, 2, 10
	22. v. 40	−	0	0
	5. vi. 40 M.H.	−	0	.
	20. vi. 40	−	0	0
	„ M.H.	−	0	.
	19. vii. 40	−	0	0
	„ M.H.	−	0	.
455	9. iii. 38	+	N.R.	N.R.
	20. x. 39	−	0	0
	16. xi. 39	+	1	5
	22. v. 40	−	0	0
	5. vi. 40 M.H.	−	0	.
	20. vi. 40	−	0	0
	„ M.H.	−	0	.
	19. vii. 40	−	0	0
	„ M.H.	+	1	.
456	4. x. 38	+	2	∞, ∞
	20. x. 39	+	2	10, 2
	22. v. 40	−	0	0
	5. vi. 40 M.H.	+	1	.
457	20. x. 39	+	2	200, 200
	22. v. 40	−	0	0
	5. vi. 40 M.H.	−	0	.
	20. vi. 40	−	0	0
	„ M.H.	+	2	.
467	18. x. 39	+	2	16, 2
	22. v. 40	−	0	0
	5. vi. 40 M.H.	+	2	.
516	21. ii. 39	+	1	80
	22. v. 40	−	0	0
	5. vi. 40 M.H.	−	0	.
	20. vi. 40	−	0	0
	„ M.H.	+	2	.
525	21. ii. 39	+	1	35
	20. x. 39	+	2	2, 4
	15. xii. 39	−	0	0
	22. v. 40	−	0	0
	5. vi. 40 M.H.	−	0	.
	20. vi. 40	−	0	0
	„ M.H.	+	1	.

Table 4 (*continued*)

Cow no.	Dates tested*	Result of test	No. of quarters from which *Str. agalactiae* was isolated	No. of *Str. agalactiae* colonies on Edwards's medium
540	12. iii. 39	+	1	5
	20. x. 39	+	3	5, 3, 10
	15. xii. 39	−	0	0
	22. v. 40	−	0	0
	5. vi. 40 M.H.	−	0	.
	20. vi. 40	−	0	0
	„ M.H.	+	3	.
551	12. ii. 39	+	1	120
	20. x. 39	−	0	0
	22. v. 40	−	0	0
	5. vi. 40 M.H.	−	0	.
	20. vi. 40	−	0	0
	„ M.H.	−	0	.
	19. vii. 40	−	0	0
	„ M.H.	−	0	.
556	12. ii. 39	+	1	8
	20. x. 39	+	1	10
	22. v. 40	−	0	0
	5. vi. 40 M.H.	−	0	.
587	1. v. 39	+	2	11, 50
	13. vi. 39	+	2	8, 1
	16. xi. 39	+	2	5, 1
	22. v. 40	−	0	0
609	12. ii. 39	+	2	14, 300
	20. x. 39	−	0	0
	22. v. 40	−	0	0
	5. vi. 40 M.H.	+	3	.
623	19. x. 39	+	2	3, 2
	22. v. 40	−	0	0
	5. vi. 40 M.H.	+	2	.
629	1. v. 39	+	4	300, 4, 60, 20
	13. vi. 39	+	2	2, 4
	20. x. 39	+	1	100
	22. v. 40	−	0	0
	5. vi. 40 M.H.	−	0	.
	20. vi. 40	−	0	0
	„ M.H.	−	0	.
	19. vii. 40	−	0	0
	„ M.H.	+	3	.
989	15. xi. 39	+	1	∞
	22. v. 40	+	1	∞
	5. vi. 40 M.H.	+	1	.
1286	31. x. 38	+	N.R.	N.R.
	7. xi. 38	−	0	0
	20. x. 39	−	0	0
	22. v. 40	−	0	0
	5. vi. 40 M.H.	−	0	.
	20. vi. 40	−	0	0
	„ M.H.	−	0	.
	19. vii. 40	−	0	0
	„ M.H.	+	1	.
1321	18. x. 39	+	3	7, 2, 4
	22. v. 40	+	1	300
	5. vi. 40 M.H.	−	0	.
1400	21. iii. 39	+	1	1
	21. x. 39	+	3	∞, 4, 5
	22. v. 40	−	0	0
	5. vi. 40 M.H.	+	3	.

+ = *Str. agalactiae* recovered. N.R. = Not recorded.

− = *Str. agalactiae* not recovered. M.H. = Modified Hotis test.

* Tested on Edwards's medium unless otherwise stated.

Other observations arising from the examinations

Inadequacy of reaction in Hotis test as a means of diagnosis. Of 447 streakings of incubated Hotis tubes on Edwards's medium 38, or 8·5 %, proved to be positive, although there was no change in the reaction, as shown by brom-cresol-purple. Many tubes showing an olive colour and many which were yellow, without formation of balls, proved on streaking to be positive. It is therefore unsafe to rely upon mere changes in reaction to decide which tubes are positive and which negative for *Str. agalactiae* (or even for other streptococci). The Hotis test is not to be relied upon as a specific test for this organism in milk.

Cleanliness in sampling. In most cases the taking of the samples for bacteriological examination was personally supervised. For routine work there can, in our experience, be no question of the value of care in taking samples and we can confirm the statements of Davis & McClemont (21). If the cows are groomed and washed, as on a farm producing graded milk, the milkers' hands washed after the milking of each cow, and a disinfectant added to the water for hand washing and udder washing, the numbers of miscellaneous organisms growing on the plates are very small. Inspection for the presence of streptococci in the absence of heavy growth of adventitious organisms is greatly facilitated and the detection of small numbers made easier. Thus with samples from Herd D the mean plate count of 1155 quarter samples plated on Edwards's medium was 1·5 and with 691 samples from Herd E 24·9 colonies. The range was so narrow as to warrant the unusual course of averaging plate counts. Another factor contributing to these results was undoubtedly the age of the samples, which averaged only 4 hr. Nevertheless our results also show that well-taken samples contain only very small numbers of organisms when plated up to 24 hr. after milking, even without special precautions to keep them cool during transit.

Haemolytic and non-haemolytic types of Str. agalactiae

From October 1938 to November 1939 when the herds (D and E) were in the process of cleaning up, forty-seven cows were found to be excreting the β-haemolytic type and eight the non-haemolytic type. In the second year from November 1939 to December 1940 it is interesting to note that only five cows excreted the β-haemolytic type and twenty-five the non-haemolytic type. The assessment of the significance of these figures must await the accumulation of greater numbers of results.

Relationship of number of quarters to number of cows infected

Using the enrichment technique it was found that ninety-three quarters of forty-nine cows were infected with *Str. agalactiae*, an average of nearly two quarters per cow. This figure is of the same order as those reported by Little (18), although our examinations were made in 'clean' herds.

Estimation of numbers of organisms in lightly infected samples

It would be of interest to know the numbers of streptococci in samples which would probably be returned as negative by plating in the presence of a bacteriostatic substance but which, by enrichment, could be shown to be positive.

To this end replicate dilutions in sterile separated milk were made, incubated and then streaked on Edwards's medium. Colonies were then picked off and identified. With the milk of several lightly infected animals which gave negative results on direct plating on Edwards's medium, it was shown that *Str. agalactiae* was actually present in numbers of the order of 100 per ml. If the distribution of organisms throughout the whole of the milk at a milking be assumed to be the same as in the samples examined the daily output of streptococci may be considerable, even in apparently light infections.

PART II. THE INCIDENCE OF INFECTION IN HEIFERS

Many workers on mastitis consider that the incidence in young cows is lower than in older cows and it has even been said to be so low as to be negligible. It will be recalled that in this work no animal was allowed to join the 'clean' herd after calving until she had been tested for the presence of *Str. agalactiae*. Between October 1937 and July 1940, 377 heifers were tested by Edwards's technique. Of these 296 gave negative results to at least one test in the first lactation and 196 to at least two tests. No less than 17 (or 4·5 %) first calvers, *whilst still in isolation*, gave positive results within a period of from 10 to 20 days of calving. Within the first lactation and after joining the 'clean' herd 64 heifers were proved to harbour *Str. agalactiae*—a total of 81 or about 21 %. It should be emphasized that the numbers present in many of these cases were minimal, but the suspicion is inescapable that the method of examination was responsible for failure to detect them earlier, even though due allowance must be made for the possibility of infection from the so-called 'clean' herd.

It is probable, bearing in mind the greater numbers of positive cases shown up by enrichment in comparison with Edwards's technique, that if enrichment methods had been used throughout the whole period of these investigations a greater number of young animals would have been shown to carry *Str. agalactiae*.

In addition to the possibility of calfhood infection attention may be drawn to the recent work of Sanders (22,23), who has incriminated flies—the house-fly amongst them—as vectors of *Str. agalactiae*. It is, of course, improbable that flies only are responsible for heifer infections. Ours occurred equally in summer and in winter, and when the young animals had had no contact with the milking herd.

If our experience is generally substantiated there is, as Minett mentions, urgent need for investigation of the sources of infection of young animals. We are already in the second year of an experiment designed to compare the incidence of mastitis, or rather the presence of *Str. agalactiae*, in the milk of groups of heifers raised as calves on infected and on non-infected milk. It seems that in the meantime there is good reason for careful selection of nurse cows for freedom from infection, and for the heat treatment of milk used for calf feeding to kill streptococci and incidentally other organisms such as tubercle bacilli and *Br. abortus*. In this connexion the pasteurization of separated milk or other by-products returned from factories and the use of sterilized churns should, we believe, be enforced (24). There is little doubt that proper care of young stock in this respect would be handsomely repaid.

DISCUSSION

It has been found that streptococci—*Str. agalactiae*, *Str. dysgalactiae* and *Str. uberis*—may, by the use of more searching bacteriological methods, be shown to be present in the milk of large numbers of animals which appear to be negative when the usual methods are used. It is probable that if even more stringent methods were devised and examinations at very short intervals made, the organisms could be demonstrated in the milk of a high proportion of milking cattle. These infections are mainly light, but it has been shown that light infections may persist over long periods—years, in fact. These very light infections are without visible effect upon the milk and no clots are present. If it be found that a high proportion of the milking animals, in herds generally, harbour *Str. agalactiae* in small numbers, it is unlikely that eradication procedures based on any but the most searching bacteriological examinations at very frequent intervals will at present succeed. It is probable that no bacteriological methods will suffice in the field, partly because of the difficulty of applying them under field conditions to large numbers of animals and partly because the results suggest that, even though the theory of contagion be admitted, there must be other methods by which infection with *Str. agalactiae* is acquired. This is the contention of a number of authors including Minett, and Hucker (25) has demonstrated the presence of streptococci in udder tissue before calving. Sanders has shown that the common house-fly and other flies may carry infection. This, however, is only a method of contagion other than by the hands of milkers.

Calfhood infection, with latency until calving, is certainly suggested, as we have found that 21 % of animals in their first lactation in herds under control and 4·5 % of heifers whilst still in isolation harboured *Str. agalactiae*. Before calving they had never, since suckling, been in contact with animals in milk. It is obvious therefore that the question of calfhood infection must be investigated before the modes of primary infection can be asserted with confidence. In the meantime, it seems that proper cowshed hygiene and management must receive more attention than is usual. Special care must be

taken to avoid injury to the udder as, if it be conceded that streptococci are likely to be present in a high proportion of animals, clinical infection is likely to follow. We have observed several such cases in animals, which on examination by ordinary bacteriological methods appeared to be free.

Although many of the animals examined have been shown to harbour *Str. agalactiae* the herds have been remarkably free from clinical mastitis. It appears from our results that frequent examinations by Edwards's technique, now shown to fail to reveal small numbers of organisms in many cases, will succeed in reducing the numbers of animals giving positive results by the enrichment technique to small proportions. A good measure of *control* is therefore possible, but it will be necessary to continue the observations over a considerably longer period before the probability of infections reaching the vanishing point can be asserted. The exact practical significance of the presence in the latent or commensal form of small numbers of presumably potentially pathogenic streptococci is not clear. It is nevertheless to be emphasized that regular bacteriological examination coupled with segregation and proper cowshed hygiene has in our case been successful in reducing clinical mastitis due to *Str. agalactiae* to very small dimensions. If the 'carrier state' is widespread, management, hygiene and avoidance of injury to milking cows assumes a new significance. Thus we have encountered several cases where clinical mastitis in entire herds has followed some irregularity in the use of milking machines.

A technique such as we have used is, of course, difficult to apply on a large scale, and the enrichment method is perhaps not the best which could be devised. But it has been shown that when the Edwards's technique is used more frequently than is usual, good results, as judged by the enrichment technique used simultaneously, are obtained. As with other biological tests, frequency and regularity of use is the essence of success. Therefore for large-scale work, at least in the early stages, other tests of even less accuracy, but used very frequently, may be of considerable value. Such tests as the 'strip cup' fall into this category, and in the absence of laboratory facilities it is probable, if it were used regularly once daily and if the results were acted upon at once, that notable inroads upon the spread of massive infection would be made. As in our experience staphylococcus infection is far more frequently responsible for the appearance of clots in the milk than is generally realized, it would be necessary to make bacteriological examinations before treatment of cases.

It is obviously desirable to prevent the passage of infection from animal to animal even though contagion be not the only mode of infection. For this purpose animals should continue to be segregated when found to be positive. Swabs, hands and teat cups should be disinfected with a solution of hypochlorite of sufficient strength (26). Although infection in calfhood, persisting until calving, has not yet been proved, the presence of *Str. agalactiae* in such a high proportion of young cows at least suggests it. In any case it is obviously

desirable to raise calves on milk which is free, not only from streptococci, but from all other pathogenic organisms (27).

SUMMARY

In the course of attempts on a field scale to eradicate or control *Str. agalactiae* infections, comparisons of the efficiency of several bacteriological methods have been made. It has been found that by using a method of enrichment, many cases of infection with small numbers of *Str. agalactiae* can be demonstrated in animals which give negative results by other methods. The frequency of examination has been shown to have an important influence upon the number of positive cases discovered.

Light infections have been proved to persist for long periods. Light infections with *Str. dysgalactiae* and *Str. uberis* have also been found in many cases. *Str. agalactiae* has been shown to be present in no less than 21 % of animals in their first lactation and 4·5 % of heifers were found to harbour this organism whilst in isolation before joining the herd. The bearing of these findings on the theory of contagion in *Str. agalactiae* infections has been discussed.

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281. VOLATILE ACIDS OF CHEESE

III. APPLICATION OF THE EXTRACTION METHOD

By E. R. HISCOX, J. HARRISON and J. Z. WOLF
National Institute for Research in Dairying, University of Reading

(With 2 Figures)

The extraction method already outlined(1) has been used in a study of the volatile acid content of various types of cheese. In a previous paper reasons for the adoption of this method were given, but working details were not included. There were points that needed further consideration before a satisfactory procedure could be put forward. Some of these points have been studied and the findings incorporated in the method at present in use, details of which are given at the end of this paper.

The method, which consists primarily in extracting the cheese with water, is based on the recognition of the varying solubilities of the volatile fatty acids in water and fat. Most of the lower acids, being water-soluble, will be removed in the water, the higher, being only slightly soluble in water but readily soluble in fat, will be transferred with the fat to the ether solution. A rough separation of the volatile fatty acids is thus accomplished at the outset before steam distillation is attempted. The distillate from the water extract should contain the bulk of the acetic acid with butyric, caproic and caprylic acids in decreasing proportions. The distillates from the ether solution of the fat should contain practically all the capric and caprylic acids and the others down to acetic acid in decreasing proportions. Lipolysis of fat in the cheese would thus be indicated by an increase in the fat-soluble acids and therefore in the total volatile acid content of the fat fraction. In mature cheese, especially those of the blue-veined type, water-insoluble volatile acids may be present in appreciable quantities in the form of water-soluble salts. These would be included in the water fraction which would then contain a disproportionate quantity of these acids. An attempt was made to avoid their inclusion by bringing the cheese to *p*H 2 by the addition of H_2SO_4, so converting these salts into acids before extracting them from the cheese. [Windisch(2) used a procedure similar to this when demonstrating breakdown of the fat during cheese ripening. The fat separating from an acidified cheese mush provided a reliable medium for analysis, since with the exception of a little butyric it retained the free fatty acids.] The volatile acid content of the fat fraction was usually increased by this treatment, but the total value for the acidified cheese was the same as or, more frequently, slightly less than the value for the untreated cheese (cf. Table 1). There were thus indications of a possible loss of free volatile acids

during the preliminary treatment. Meanwhile a promising method for the estimation of the various acids in the distillates was being studied, and since such changes in the preliminary rough separation would not facilitate the final estimate, the idea of acidification of the cheese was dropped as an unnecessary complication. It was decided to adhere to the simpler method of extracting the untreated cheese.

It was found that the figure for total volatile acids in the distillate from the water fraction was usually of the same order as that obtained by simple steam distillation of the cheese mush. For Stilton cheese, however, the quantity in the water fraction was frequently much higher. The acids dissolved in the fat, which should comprise the bulk of the less 'soluble' members of the series and which resulted chiefly from lipolysis were thus, presumably, not accounted for by the steam distillation method. This statement is supported by the fact that the fat of a Cheddar and of a Stilton cheese separated in the

Table 1. *Volatile acids as ml. N/10 acid per 100 g. of cheese*

		Extraction method					
		Normal *p*H			Brought to *p*H 2		
Type of cheese	Simple steam distillation	Water fraction	Fat fraction and residues	Total	Water fraction	Fat fraction and residues	Total
Roquefort (1)	70·15	74·50	64·05	138·55	63·15	73·35	136·50
Roquefort (2)	70·90	72·25	40·80	113·10	54·30	51·70	106·00
Danish blue (1)	68·65	62·15	77·45	139·60	52·05	81·80	133·85
Danish blue (2)	64·90	60·90	45·05	105·95	53·55	53·90	107·45
Gorgonzola (1)	22·75	26·95	47·30	74·25	30·20	48·00	78·20
Gorgonzola (2)	19·15	23·00	40·05	63·05	22·40	43·80	66·20
Stilton	12·50	21·75	24·40	46·15	13·25	31·75	45·00
Portuguese sheep's milk	29·75	28·65	26·65	55·30	33·20	25·65	58·85

manner suggested and steam distilled directly, i.e. without preliminary washing out of the fatty acids with alkali, gave figures for volatile acids of 3·65 and 7·15 ml. respectively, as compared with 12·5 and 27·4 ml. *N*/10 acid per 100 g. cheese obtained by distilling the alkali washings. The bulk of the acids was retained by the fat and could not be released by direct steam distillation. When the protein residues were steam distilled directly, i.e. without preliminary extraction with ether, the figures obtained for Cheddar cheese differed only slightly from those obtained by the routine extraction procedure, but for Stilton they were definitely lower. It is probable that the degree of breakdown of the cheese protein has an influence on the retentive power. In this connexion it may be mentioned that fat and protein from a Cheddar cheese were used for the experiments reported previously (3). It is possible that fat and protein from a mature Stilton cheese would have shown greater retentivity. Lane & Hammer (4), in studying the development of volatile acids in blue cheese (Roquefort type), used direct steam distillation to demonstrate the increase in the volatile acid content during ripening. They also

noted that the acid (titratable) values for the fat also increased during ripening, but have not specified the acids responsible for this increase. No doubt a considerable proportion of this acidity was due to volatile acids produced by lipolysis, which, being dissolved in the fat, were additional to those estimated by direct steam distillation. If these were taken into consideration the total volatile acids would be markedly increased. Our values for steam distillation have been doubled in the case of Cheddar, Cheshire and cheese of the Roquefort type. For Gorgonzola the values were increased about three times, for Stilton three to five times (cf. Table 6).

A scheme suitable for routine determinations of the distribution of the various volatile acids in the distillates was still under test and could not therefore be used for the estimation of the various acids extracted from the cheese in the experiments to be described in this and following papers. Only an approximate separation based on the proportionate amounts of volatile acids in the water and fat fractions has been attempted, but this has sufficed to give a general idea of their distribution and to suggest fundamental differences in the course of the ripening process in several varieties of cheese.

During the distillation water-insoluble acids, presumably lauric and capric, which frequently deposited on the condenser, were collected on a filter paper. These 'insolubles' were dissolved in alcohol and estimated by titration with $N/10$ NaOH solution.

Emulsification of the fat with dilute alkali

During preliminary experiments, while the technique for the extraction of the volatile acids from cheese was being developed, considerable difficulty was sometimes experienced while washing the fat with dilute alkali. During the shaking the fat emulsified, and it was necessary to break the emulsion before the separation of the fat from the alkali washings could continue. When this happened the results seemed to be abnormally high, and there was often a considerable difference between duplicate samples one of which had been emulsified by vigorous shaking with the alkali. Control experiments showed that a breakdown of the fat occurred during prolonged contact of the emulsion with dilute alkali and that the total amounts of volatile acids extracted were considerably greater from the emulsified than from the non-emulsified samples. But when acids such as acetic, butyric and caproic were added to the fat, they were less easily recovered from the emulsified samples.

Trouble due to emulsification of the fat was rarely experienced during the main tests which form the subject of following papers. The cheese examined were all young or in early stages of maturity. Over-ripe cheese had, however, often been used for the preliminary tests, and the advanced state of the fat breakdown probably accounted for the frequent tendency to emulsification then experienced. This view was strengthened by the recurrence of the

difficulty when estimating the volatile acids of cheese in which considerable fat breakdown had been induced by the addition of lipase to the cheese milk.

Normally, difficulties due to emulsification of the fat should not arise, but in all cases too vigorous shaking of the fat with alkali should be avoided. Emulsification of the fat will certainly give unreliable results.

The presence of CO_2 in the distillate

Since phenolphthalein was the indicator used for all titrations in these experiments, it was felt that the distillates should be examined for the presence of CO_2, which might well prove to be a factor of some importance.

Two possible methods of eliminating CO_2 from the distillate were considered, aerating with CO_2-free air for 20 min. (5) and boiling under a reflux condenser.

Either of these processes carried out before titration would remove CO_2. The efficiency and practicability of the two methods were compared by dividing a quantity of distillate into six parts and testing both methods in duplicate.

The results appeared to be satisfactory, but confirmatory tests were made on solutions of volatile fatty acids of known strength, through which CO_2 had been bubbled. The figures are given in Table 2.

Table 2. *Titration values expressed in ml. of N*/10 NaOH

	Direct titration	Titration after 20 min. aeration	Titration after 15 min. refluxing
500 ml. portion of distillate	1·95	1·70	1·60
	1·95	1·65	1·64
100 ml. portion of butyric acid solution:			
(1) Original solution	6·56	6·52	6·37*
	6·58	6·53	6·49*
(2) After adding CO_2	7·84	6·55	6·62
	7·91	6·56	6·61

* Condenser rinsings not titrated.

Similar tests with other solutions of butyric and acetic acids gave comparable results. Both methods appeared to be equally effective in removing CO_2 and avoiding loss of volatile acids. It was therefore decided to incorporate the more easily manipulated aeration into the extraction method.

In the various series of cheese examined for volatile fatty acids one distillate from each group of duplicate, triplicate or quadruplicate samples was well mixed and divided into two parts, one of which was subjected to 20 min. aeration before titration. In this way varying amounts of CO_2 were detected in the distillate. The amounts for the water fraction, fat fraction and protein residues were added together and the totals are given in Table 3 and Fig. 1.

Control distillations showed that the amount of CO_2 carried over from the NaOH solution was very small and could not account for the relatively large quantities demonstrated in some of the distillates. It is more probable that the bulk of the CO_2 was derived from the cheese itself. This gas is evolved

Table 3. *Variations in amount of* CO_2 *(as ml. N/10 NaOH per 100 g. cheese) during ripening*

	Age in days ...	0–1	2–3	4–5	6–7	8–9	12	15
Young cheese								
CO_2 in 100 g.	Cheddar	8·50	5·50	4·75	6·75	7·25	6·25	7·00
	Stilton	5·00	3·25	6·00	9·25	5·75	8·25	7·25
	Age in days ...	18	21	26	34	42	57	83
CO_2 in 100 g.	Cheddar	6·75	6·25	6·00	6·50	—	13·00	11·50
	Stilton	6·25	6·00	9·75	7·00	16·75	13·75	—
	Age in days ...	76	82	90	103–104		118–19	132
Maturing Stilton cheese								
CO_2 in 100 g.	Unpricked	—	13·00	—	6·50		4·00	—
	Pricked at 63 days	15·00	—	11·50	15·50		6·00	8·25
	Age in days ...	147	155	167	183		195	238
CO_2 in 100 g.	Unpricked	5·75	11·00	14·00	15·25		18·50	16·50
	Pricked at 63 days	0·50	—	—	—		—	—

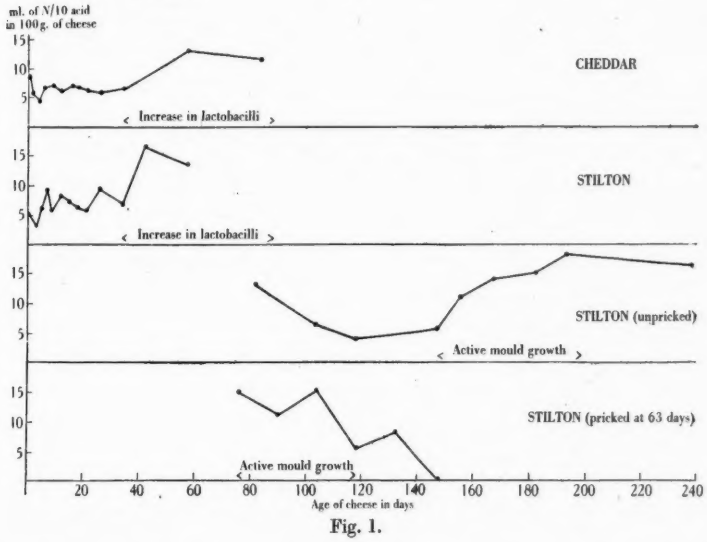

Fig. 1.

during the growth of micro-organisms, and some would, no doubt, accumulate in the body of the cheese.

Although the figures varied from cheese to cheese it seems that the general trend was towards an increase in the amount of CO_2 during periods of active growth of micro-organisms within the cheese. During periods of active bacterial and mould growth it is possible that the production of CO_2 takes place more rapidly than its diffusion from the cheese and so accumulations

occur. Later, when the rate of growth declines the rate of diffusion of CO_2 may exceed its rate of production and these accumulations would disappear.

The comparatively rapid decrease in the amount of CO_2 in the pricked cheese, illustrated in Fig. 1, may be related to the greater ease of diffusion through the prick-holes.

Presence of lactic acid in the distillates of cheese

It has been shown that lactic acid is slightly volatile in steam (Allen & Harrison(6)), and since it is present in cheese it may also be found in the distillates.

In order to estimate the possible error in titre arising from the presence of lactic acid, estimations of the quantity present were made on various distillates using the technique of Friedemann *et al.*(7).

The amount of lactic acid in the distillate was estimated separately for each of the three fractions, when it was found that the bulk of the lactic acid was in the distillate from the water fraction. The amounts in the distillates from the fat and protein residues were very small.

The average figures for three samples of Cheddar cheese are given in Table 4.

Table 4. *Lactic acid expressed as ml. N/10 acid per 100 g. cheese*

Simple steam distillation	Distillates from: Water fraction	Fat fraction	Protein residues	Total by extraction method
0·95	2·60	0·20	0·20	3·00

This amount is very small in comparison with the usual total of 40–80 ml. $N/10$ acid in the distillates from Cheddar cheese. Corrections for lactic acid have, therefore, been omitted in this work.

Effect of light upon the minced cheese samples

Portions of minced cheese exposed to full daylight on the bench developed a tallowy taste and smell within a few hours, presumably as a result of light-catalysed oxidation of the fat. It seemed possible that a similar reaction might occur during the preliminary treatment of the samples under investigation for volatile acids with consequent errors in the results. A number of parallel determinations were therefore made in which the samples were either exposed to full daylight or kept in the dark throughout the extractions. No difference in the amount of volatile acids distilling over was observed, nevertheless it was thought advisable to avoid unnecessary exposure of the samples to bright daylight and high temperatures at any stage of the process.

Experimental methods

The procedure finally adopted is given below.

Sampling. A wedge-shaped section cut to the centre, from top to bottom of the cheese, was taken in an effort to obtain a representative sample. For

Stilton and Roquefort one-quarter of the cheese was taken in this way; for the larger types a section weighing at least 2 lb. was used. The coat was removed and the body of the cheese finely minced and thoroughly mixed. The amount of mixing necessary to obtain a uniform sample varied with the type and maturity of the cheese. Great difficulty was experienced in obtaining a uniform sample from well-blued mature Stilton.

Direct steam distillation. 20 g. of the cheese mixture were mixed with CO_2-free distilled water, acidified to *p*H 2 with H_2SO_4, the volume made up to 100 ml. and the mush steam distilled at constant volume, using the standard apparatus previously described (2). 500 ml. of distillate were collected and titrated with *N*/10 NaOH solution. (In some cases $Ba(OH)_2$ solution was used.) Estimations were made in duplicate.

Extraction method. 20 g. of cheese mixed in a centrifuge bottle (250 ml. capacity) with 100 ml. of CO_2-free water at about 40° C. were shaken for 3 hr. on a mechanical shaker. Usually, most of the fat was churned into balls which were easily removed and dissolved in ether. The jar and its contents were then centrifuged and cooled, if necessary, to harden the floating fat layer which could then be transferred to the ether solution. The supernatant liquid was poured into a flask leaving the residues for further washing. The sample was washed three times in this way, a short shaking by hand being used for the second and third washings. Determinations were made in duplicate or, more frequently, in quadruplicate.

Water washings of the cheese. The three quantities of water washings were bulked (300 ml.), acidified to *p*H 2 and steam distilled, 1500 ml. of distillate being collected. A small filter was placed at the mouth of the receiver to collect water-insoluble deposits. At the end of the distillation, residues in the condenser and filter were washed with 10 ml. of ice-cold water. The deposits were then dissolved in alcohol and titrated separately.

Alkali washings of the ether solutions of fat. The ether solution of fat was neutralized with strong (20 %) NaOH solution, then transferred to a separating funnel where it was washed six times with 50 ml. quantities of *N*/10 NaOH solution. The ether was driven off from the washings (300 ml.), which were then brought to *p*H 2 and steam distilled—1500 ml. of distillate were collected and the deposits in the condenser and filter treated as described above.

Alkali washings of the ether extract of the residues. The protein residue in the centrifuge jar was mixed with sufficient acid-washed sand to make it friable, acidified to *p*H 2 and extracted in a Soxhlet thimble continuously for 36 hr. (water-bath at 40–45° C.). The ether extract was neutralized, and washed four times with 50 ml. quantities of *N*/10 NaOH solution. The washings (200 ml.) were freed from ether and steam distilled as before, except that only 1000 ml. of distillate were collected.

The acids extracted from these residues were usually small in quantity and predominantly fat-soluble. In computing results the figures have been added to those of the fat fraction.

Error due to CO_2. The individual distillates from one sample of each cheese were well mixed, then divided into two equal portions through one of which CO_2-free air was passed for 20 min. to remove any CO_2 dissolved in the liquid. The error that might be due to the presence of CO_2 was thus determined.

A rapid method for the separation of the volatile fatty acids in cheese distillates

Butter-fat has been shown to have a marked retentive effect on the volatile fatty acids(2). Since it forms a considerable proportion of the mass of the cheese it is natural to assume that it affects the final relative distribution of the fatty acids in the fat and water fractions in the method of extraction just described.

For this purpose it is necessary to determine the distribution coefficient of the fatty acids between fat and water.

Experimental. It was considered essential to have the butter-fat in liquid form in order to allow thorough mixing of the two phases and to attain true equilibrium. A temperature of 55° C. was selected, since the dispersed fat globules rose rapidly to the surface after each shaking. Fresh butter-fat from the mixed milk of a Guernsey herd, pasteurized to destroy lipase, was melted at 55°, the fat decanted off and filtered through a thick filter paper. From this bulk 50 ml. (44·40 g.) were run out from a jacketed burette, maintained at 55° C. by a circulating stream of warm water, into a separatory funnel and 50 ml. of $N/10$, $N/20$, or $N/100$ solution of a selected fatty acid added. The acids were the purest commercially obtainable (Kahlbaum) and solutions were prepared in CO_2-free water. Preliminary experiments had shown that after a single shaking and standing for 30 min. equilibrium had been reached. Nevertheless a longer time of contact was considered desirable.

It was found that after 2 hr. in the 55° C. incubator the contents of the funnel had reached the required temperature. The funnel was now shaken for 2 min., returned to the incubator, and shaken again after an hour. At the end of a further hour the aqueous portion (approximately 50 ml.) was run off and 20 ml. aliquot portions titrated with standard soda. All experiments were carried out in duplicate and some in quadruplicate.

Summer and winter butter-fats of the same herd were compared. No differences were observed in the constants obtained, maximum discrepancies in titrations being 0·05 ml. Corrections were made for the acidity of the butter-fat. Suitable controls of butter-fat, shaken with water, were included with each experiment to obtain the acidity due to the fat. This figure was subtracted from the titrations.

Table 5 summarizes the results obtained. It will be seen that, within a very narrow range, the distribution of each acid between water and fat was independent of the concentration used. These figures do not markedly differ from those obtained by Lane and Hammer(8), who used a temperature of 7·2° C. and a brine solution instead of water.

The solubility of the fatty acids in fat increased with increasing molecular weight of the acid, and for caprylic and higher members of the series solubility in the fat was almost complete. This suggested that the distribution of the fatty acids in butter-fat might offer a basis for a separation of the lower members of the series from the higher, so providing a rapid means of estimating the amounts of the various volatile fatty acids in cheese distillates. When a series of mixtures of aqueous solutions of fatty acids was shaken with butter-fat it was found that each acid was taken up in the proportion that would be expected from its distribution factor, the quantity of each being unaffected

Table 5

		Distribution factors obtained by shaking 50 ml. of aqueous solution of acid with 50 ml. of butter-fat at 55° C.		Values of Lane & Hammer(8) carried out at 7·2° C.	
		% appearing in		% acid retained in	
Acid	Normality	Water	Fat	Fat	NaCl solution
Acetic	0·1184	95·86	4·14	2	98
	0·0594	96·53	3·47		
	0·010	95·20	4·80		
	Mean (nearest whole no.)	96·00	4·00		
Propionic	0·1049	86·74	13·26	14	86
	0·0462	87·17	12·83		
	0·01	85·52	14·48		
	Mean (nearest whole no.)	86·00	14·00		
Butyric	0·1005	63·10	36·90	41	59
	0·050	64·96	35·04		
	0·010	64·87	35·54		
	Mean (nearest whole no.)	64·00	36·00		
Caproic	0·050	11·36	88·64	91	9
	0·010	13·25	86·75		
	Mean (nearest whole no.)	12·00	88·00		
Caprylic	0·0036	1·36	98·64	99	1

by the presence of the other acids. The following two examples chosen out of numerous experiments illustrate the point.

Mixture A:

20 ml. butyric acid equivalent to 19·65 ml. *N*/10 NaOH.
20 ml. caproic acid equivalent to 9·24 ml. *N*/10 NaOH.
10 ml. acetic acid equivalent to 11·89 ml. *N*/10 NaOH.
The above mixture (50 ml.) was shaken up with 50 ml. of butter-fat.
20 ml. of aqueous phase was equivalent to 9·98 ml. *N*/10 NaOH.
50 ml. of aqueous phase contained 24·95 ml. *N*/10 acid.
Correction for acidity of butter-fat 0·02 ml. *N*/10 acid.

From the factors of Table 5 it will be seen that
96·00 % of acetic acid or 11·41 ml. *N*/10 should remain in the water.
64·00 % of butyric acid or 12·58 ml. *N*/10 should remain in the water.
12·00 % of caproic acid or 1·11 ml. *N*/10 should remain in the water.
Total acid in the water: 24·93 ml. *N*/10 acid (experimental), 25·10 ml. *N*/10 acid (theoretical).

Mixture B:

By dissolving the fat in ether after use, shaking with dilute soda, and washing the fat twice with warm water, the dissolved acids are removed, and the butter-fat may, if necessary, be used again.

Butter-fat reconditioned in this way was used in the following experiment.

The acid mixture consisted of

20 ml. acetic acid equivalent to 19·25 ml. *N*/10 NaOH.

5 ml. butyric acid equivalent to 4·80 ml. *N*/10 NaOH.

25 ml. distilled water.

The above mixture (50 ml.) was shaken up with 50 ml. of the 'reconditioned' butter-fat.

5 ml. of aqueous phase was equivalent to 2·16 ml. *N*/10 NaOH.

50 ml. of aqueous phase contained 21·6 ml. *N*/10 acid.

Correction for acidity of butter-fat 0·02 ml. *N*/10 acid.

From the factors of Table 5 it will be seen that

96·00 % of the acetic acid or 18·48 ml. *N*/10 acid will remain in the water.

64·00 % of the butyric acid or 3·07 ml. *N*/10 acid will remain in the water.

Total acid in the water: 21·58 ml. *N*/10 (experimental), 21·55 ml. *N*/10 (theoretical).

The separation of mixtures of the fatty acids has been attempted in various ways. Thus Orla-Jensen (9) used fractional precipitation of the silver salts, while Hammer & Sherwood (10) found the barium salts preferable. Neither of these is, however, suitable as a rapid routine method for the quantitative estimation of the acids. The more recent method of Friedemann (5) would seem to be accurate but is nevertheless lengthy. It appeared to us that much information on the composition of a cheese distillate could be obtained simply by shaking it with butter-fat and carrying out a Duclaux distillation on the aqueous portion. With a fat:water ratio of 1:1 about 12 % of caproic would remain in the water. Usually this quantity would be too small to interfere seriously with the Duclaux distillation. But it could be reduced still further by increasing the fat:water ratio.

In practice the bulk of the capric and lauric acids would be retained on the filter paper during the distillation and, after solution in alcohol, could be estimated by titration. If the distillate were then shaken with butter-fat, the fat would take up the remaining traces of capric, the caprylic, the caproic and a large proportion of the butyric acids. The remaining acids (butyric, propionic, acetic or formic) would be contained in the water phase and their proportions could be estimated by means of Duclaux distillations. The total quantities of all the acids in the original distillate could then be computed from distribution tables.

To effect this separation the original neutralized distillate must be reduced to a small volume. In our experiments it was found that after evaporation and redistillation at constant volume, the full quantity of volatile acids in the original distillate was never recovered. The loss was usually greater than would have been expected from the presence of small quantities of lactic acid in the original distillate (5). There were probably other contributory causes, some of which are now under consideration.

Application of the extraction method

The extraction method just described has been used in the study of the volatile fatty acids of several types of mature cheese (white-pressed and blue-veined), and of the changes in the quantity and distribution of the volatile acids during the ripening of Stilton and Cheddar cheese. Other analytical data were also obtained for these samples and some have been included in Table 6. Figures for the mature cheese only are shown in this table; data relating to the changes in the distribution of volatile acids during the ripening processes will be discussed in a subsequent paper.

The nitrogen breakdown was determined by Davies's[1] method using trichloracetic acid (4 %) as the precipitant for the estimation of the non-protein nitrogen (11), the *p*H by means of the quinhydrone electrode and the volatile acids by both direct steam distillation and the extraction method. No correction for the presence of CO_2 in the distillates has been made in the figures in this table.

Most of the Stilton, the Cheddar and other white varieties of cheese were made in the experimental dairy at the Institute and were under observation throughout the ripening period. They were taken from the cheese store at the time of analysis and had not therefore been exposed to the exigencies of commercial conditions. Others, a Stilton, a Cheddar and a Cheshire cheese, were from first-prize winning groups at the London Dairy Show, and the Portuguese sheep's milk (serra) cheese were received direct, but after some delay in transit, from Portugal by courtesy of Dr M. Moreira Jacob. No information was available as to the previous history of the Roquefort, Danish blue and Gorgonzola cheese, but they were bought from a reliable store as typical market specimens of these varieties. All the cheese were sound, of good flavour and, with the exception of R_2 (Roquefort), D_2 (Danish blue) and II C_2 (Cheddar), were well-matured. R_1 (Roquefort) and III S_{12} (Stilton) were generally considered slightly over-ripe, II C_3, IV C_2 and IV C_3 were considerably older and more full-flavoured than Cheddar would generally be when consumed, and III S_{167} and I S_{146} were abnormal Stiltons, since they, unlike other members of groups III and I, had been heavily pricked.

The figures in Table 6 illustrate the great influence exerted on the ripening process by the strongly caseolytic and lipolytic mould of the blue-veined varieties. The differences were most marked in the percentage of ammonia nitrogen, the *p*H, and the quantity and distribution of volatile fatty acids.

The figures also show that the methods of manufacture and after treatment of blue-veined cheese influence the chemical activities of the mould. Blue-veined cheese are not all of one category. There were essential differences in the chemical constitution of Roquefort and Danish blue cheese on the one hand

[1] We are indebted to the late Dr W. L. Davies for the data on the nitrogen breakdown and the moisture content.

Table 6

Cheese		Moisture g./100 g. of cheese	Protein breakdown in % of total nitrogen			Acidity *p*H	Volatile acids—ml. *N*/10 acid in 100 g. of cheese				
								Extraction method			
Code number	Description		Non-protein nitrogen	Non-protein Sorensen nitrogen	Ammonia nitrogen		Direct steam distillation	Total	Water fraction	Fat fraction	Water insoluble
R_1	Roquefort, over-ripe	38·23	51·9	25·8	8·7	5·77	70·1	138·6	74·5	64·1	—
R_2	Roquefort, less ripe	36·33	28·3	14·0	4·3	5·32	70·9	113·1	72·3	40·8	20·6
D_1	Danish blue, ripe	39·18	26·9	20·9	6·4	5·21	68·7	139·6	62·1	77·5	63·6
D_2	Danish blue, less ripe	39·59	38·5	11·4	4·6	5·21	64·9	106·0	60·9	45·1	25·6
III S_{167}	Stilton, heavily pricked, 110 days	36·1	52·03	15·4	9·5	5·39	12·3	83·8	55·3	27·6	24·2
I S_{146}	Stilton, heavily pricked, 84 days	31·28	36·0	14·6	7·6	6·0	17·7	92·1	34·9	57·8	42·1
G_1	Gorgonzola, well-blued	44·9	44·4	14·5	7·9	6·02	22·8	74·3	27·0	47·3	—
G_2	Gorgonzola, well-blued	41·13	37·9	14·7	7·2	6·12	19·2	63·1	23·0	40·1	26·1
S N_3	Stilton, unpricked, blue, 186 days	34·1	31·8	10·8	7·8	5·97	—	70·2	33·1	37·1	26·6
S L_1	Stilton, unpricked, blue, 186 days	33·6	31·1	11·1	6·5	5·69	—	68·7	26·8	41·9	33·2
III S_{12}	Stilton, unpricked, blue, 238 days	32·5	43·2	20·8	9·5	6·4	18·3	66·6	25·7	40·9	26·6
III S_{11}	Stilton, unpricked, blue, 195 days	33·1	40·8	16·4	7·9	6·07	15·3	56·3	20·2	36·1	17·4
III S_{10}	Stilton, unpricked, blue, 183 days	33·0	37·1	12·4	6·6	6·23	11·2	61·5	21·9	39·6	22·4
S H	Stilton, well blued	—	—	—	—	—	11·8	53·1	25·0	28·1	—
S DS	Stilton, prize-winning cheese, 147 days	—	—	—	—	—	15·5	50·8	19·5	31·3	16·5
I S_9	Stilton, lightly pricked at 2 months	27·4	31·9	12·5	6·5	5·72	11·6	37·9	13·8	24·2	15·5
I S_8	Stilton, lightly pricked at 132 days	28·8	34·3	12·5	6·4	5·81	9·8	36·2	14·0	22·3	15·4
S N_6	Stilton, unpricked, blue, 186 days	36·3	33·4	14·0	8·0	6·03	—	27·3	11·9	15·4	6·2
S N_{24}	Stilton, unpricked, blue, 186 days	35·2	31·3	12·1	6·8	5·79	—	29·9	13·3	16·6	8·2
S L_{21}	Stilton, unpricked, blue, 186 days	32·4	36·8	14·8	8·0	5·92	—	30·6	13·7	16·9	7·3
S L_{22}	Stilton, unpricked, blue, 186 days	32·0	36·9	14·3	7·9	5·83	—	27·9	11·3	16·6	6·4
S Wh	Stilton, non-blued, 130 days	28·1	10·8	4·7	2·4	4·69	15·4	54·7	20·6	34·1	—
P	Portuguese sheep's milk	46·45	16·5	3·1	0·99	5·25	29·8	61·3	34·7	26·7	—
P_1	Portuguese sheep's milk	—	18·5	6·8	2·4	5·29	—	53·8	36·8	17·0	3·9
P_2	Portuguese sheep's milk	—	19·1	5·3	3·0	5·13	—	52·9	38·2	14·6	4·5
La_1	Lancashire, 2 months	—	20·6	5·0	1·3	4·99	31·28	42·0	31·8	10·2	7·4
La_2	Lancashire, 4 months	—	24·5	8·0	1·9	5·04	34·64	44·7	34·9	9·8	5·5
Ch_1	Cheshire, 4 months	—	28·5	11·3	—	—	—	—	—	—	—
Ch_2	Cheshire, 132 days	—	26·3	9·2	3·1	5·19	—	—	—	—	—
Ch DS	Cheshire, prize-winning cheese	—	—	—	—	—	28·8	58·8	38·0	20·8	5·0
L_1	Leicester, 4 months	—	28·3	9·6	—	—	—	—	—	—	—
L_2	Leicester, 132 days	—	28·2	9·0	2·9	5·10	—	—	—	—	—
C_1	Cheddar, 4 months	—	25·7	9·8	—	—	—	—	—	—	—
C_2	Cheddar, 132 days	—	23·3	6·9	2·3	5·10	—	—	—	—	—
C S_6	Cheddar, from same milk as S Wh, 130 days	34·19	19·4	6·6	2·4	5·23	35·5	77·2	41·8	35·5	—
C DS	Cheddar, prize-winning cheese	—	—	—	—	—	36·3	67·3	43·3	24·0	7·8
II C_2	Cheddar, 57 days	34·6	15·3	3·7	0·4	4·76	15·0	34·3	18·6	15·8	—
II C_3	Cheddar, 205 days	29·2	24·3	8·8	1·8	4·87	47·0	78·4	49·1	29·3	8·5
IV C_2	Cheddar, 224 days	33·7	27·9	14·8	3·7	—	105·0	133·0	104·3	28·8	6·6
IV C_3	Cheddar, 294 days	33·7	33·8	15·4	4·8	—	142·6	166·6	143·8	22·9	8·9
V C_{13}	Cheddar, 83 days	36·0	22·1	9·7	2·1	5·13	38·0	62·0	42·0	20·0	7·5
C L_4	Cheddar	—	—	—	—	—	—	46·1	32·5	12·5	8·7

and of Gorgonzola and Stilton on the other, such as might be expected from the earlier and more prolonged mould activity in the former group.

The retentivity for volatile fatty acids varied with the type of cheese. It was greatest for Stilton and Gorgonzola in which the quantities of total volatile acids as estimated by the extraction method were three to five times those estimated by means of direct steam distillation. For all other types of cheese included in this table it rarely exceeded twice the quantity.

Another point of interest, more clearly illustrated by Fig. 2, was that, for Stilton and Gorgonzola cheese, the proportion of volatile acids in the distillate from the fat fraction was higher than that from the water fraction. The reverse was found for the white varieties and blue cheese of the Roquefort type, with the exception of D_1.

The true significance of this difference might be more easily suggested were details of the distribution of the volatile acids available for all these cheese. It does, however, strongly indicate fundamental differences in the course and the degree of the fermentation of the sugar and fat in the three groups of cheese. In white cheeses, such as Cheddar, there appears to be very little lipolysis and the water-soluble acetic acid (not derived from fat) constitutes the bulk of the volatile acids. This is also true of young white Stiltons, as will be shown in a later paper, but with the development of the mould, lipolysis assumes an increasing importance.

Orla-Jensen (12) has reported appreciable quantities of butyric acid in Roquefort cheese and our preliminary experiments have indicated that butyric acid is present in the distillates from Roquefort and Danish blue cheese in much larger quantities than in the distillates from Stilton. Because the partition coefficient of butyric acid is higher for water than for butter-fat, a high proportion of the total bulk would be found in the water fraction.

The Stilton cheese included in Table 6 were in early stages of maturity. With increasing age the quantities of volatile acids increased, as in III S_{12} for example, but the balance between the acids in the water and fat fractions remained unchanged for some time. Whether this balance would have been reversed had the cheese been allowed to become much over-ripe was not determined, but artificial hastening of the ripening of III S_{167}, a cheese of the same batch, by means of heavy and repeated pricking, led to an increase in the total volatile acids, a reversal of the balance between the water and fat fractions and a lowering of the *p*H. In these respects this cheese resembled Roquefort, and moreover its flavour was reminiscent of this variety. This gives a strong indication of the danger to the quality of Stilton cheese of indiscriminate pricking to ensure and hasten the mould development. A light and judicious pricking, such as I S_8 and I S_9 received, appeared not to detract from the quality, but another cheese, I S_{146}, from the same batch, ripened rapidly by heavy pricking, was of poorer quality, with a high volatile acid content, although the balance of the water and fat fractions and the *p*H were normal. If it is desired to retain the distinctive characters of Stilton cheese,

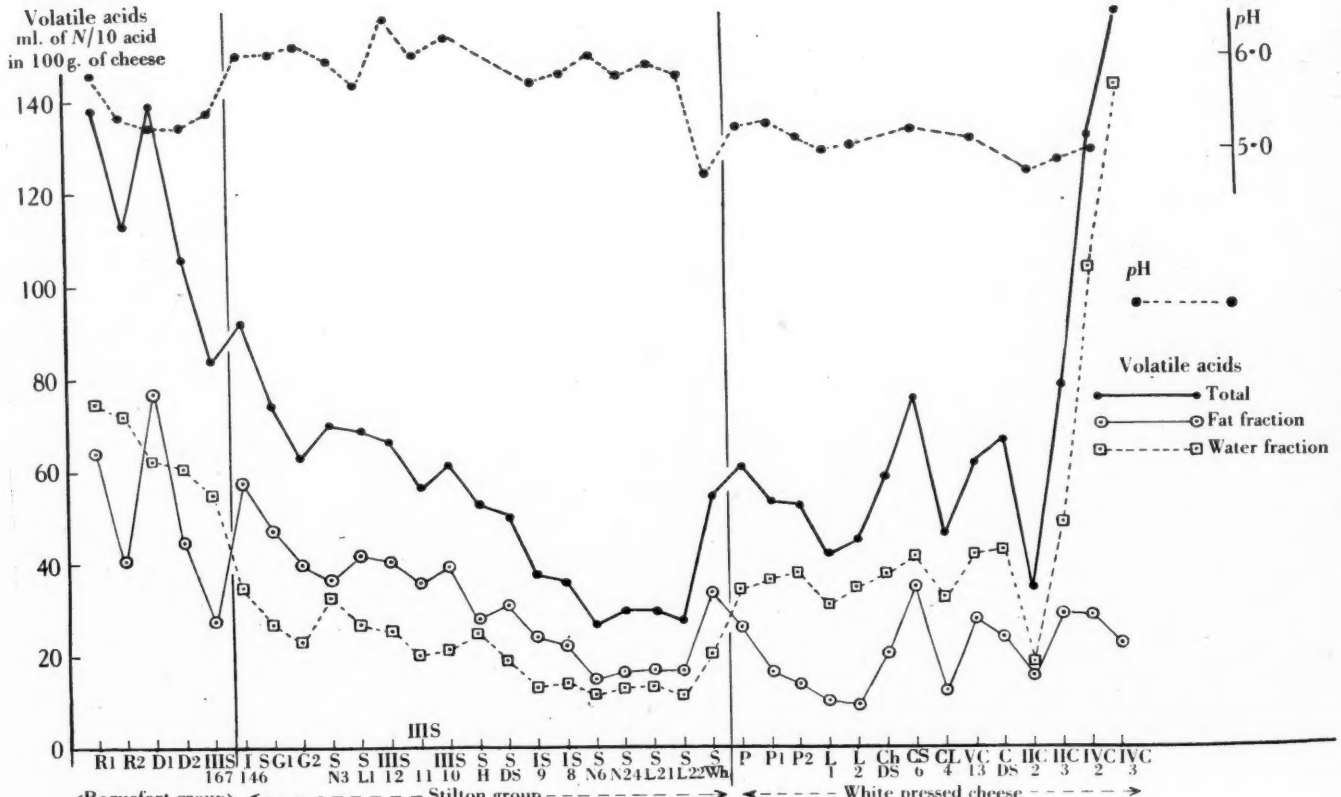
Volatile acids
ml. of N/10 acid
in 100 g. of cheese
140
120
100
80
60
40
20
0
pH
6·0
5·0
pH
Volatile acids
Total
Fat fraction
Water fraction
IIIS
R1 R2 D1 D2 IIIS I S G1 G2 S S IIIS IIIS S S IS IS S S S S S P P1 P2 L L Ch CS CL VC C IIC IIC IVC IVC
167 146 N3 L1 12 11 10 H DS 9 8 N6 N24 L21 L22 Wh 1 2 DS 6 4 13 DS 2 3 2 3
<Roquefort group> Stilton group White pressed cheese

Fig. 2.

pricking, which may be advantageous in some circumstances, must be moderate in extent, and not carried out at too early a stage of the ripening period.

SUMMARY

1. Details are given of the extraction method for the estimation of the volatile acids in cheese, as used in the study of some white and blue-veined varieties.

2. On the basis of the distribution of volatile acids, hydrogen-ion concentration and nitrogen breakdown, distinctions could be drawn not only between the white and blue-veined varieties as a whole, but also between the Roquefort and Stilton groups of the latter varieties.

3. A high total quantity and a characteristic distribution of the volatile acids appeared to be related to the heavy pricking of cheese of the Roquefort group.

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282. A PRELIMINARY STUDY OF THE EFFECTS OF VARYING PITCHING CONSISTENCY AND RATE OF SCALD ON THE PHYSICAL AND CHEMICAL PROPERTIES OF CHEDDAR CHEESE AND ON THE FIRMNESS OF THE CHEESE AS JUDGED BY CHEESE-MAKERS, BAKERS AND OTHERS

By G. W. SCOTT BLAIR, F. M. V. COPPEN and D. V. DEARDEN
National Institute for Research in Dairying, University of Reading

In earlier papers(1,2) the importance of assessing the correct moment at which cheese is to be pitched was stressed, a method for measuring quantitatively and rapidly the consistency of curd approaching the right condition for pitching was described and the relationship between the results of such tests and the judgements given by expert cheese-makers was discussed.

The object of the present study was to investigate quantitatively the effects of pitching at various known consistencies on the quality of the cheese and to compare the results of physical and chemical tests with those of personal judgements by various types of people.

Table 1

(*a*) Time from adding starter to

Cheese no.	Renneting hr. m.	Cut hr. m.	Heat on hr. m.	Heat off hr. m.	Pitching hr. m.	*W/h* consistency at pitching
1	1 2	1 47	2 7	2 40	2 45	20·9
2	1 2	1 47	2 7	2 42	3 3	19·0
3	1 19	2 4	2 24	—	3 15	19·7
4	1 20	2 4	2 24	3 10	3 44	17·2
5	1 10	1 55	2 15	2 40	2 52	19·8
6	1 10	1 55	2 15	2 50	3 25	18·5

(*b*) Time from pitching to

Cheese no.	Whey off hr. m.	On rack hr. m.	Acid	1st turn hr. m.	Acid	Mill hr. m.	Hoop hr. m.	Press hr. m.
1	0 30	0 38	0·225	1 20	0·33	3 30	4 15	—
2	0 30	0 42	0·25	1 22	0·43	2 46	3 32	—
3	0 20	0 38	0·25	1 14	0·40	2 43	3 58	4 13
4	0 25	0 49	0·30	1 29	No whey	3 14	3 54	4 9
5	0 20	0 38	—	1 23	0·39	3 13	—	4 13
6	0 20	0 40	0·29	1 25	0·47	3 7	—	4 5

The milk used for the present experiment was pasteurized before delivery and when received was mixed and divided into six equal portions. Each portion was inoculated with an equal amount of starter (1 %), and rennet was added when the titratable acidity was 0·025 % above the initial value of the uninoculated milk.

The size of cut, scalding temperature (100° F.) and general management of the curds were kept as nearly as possible the same for each vat of milk. The pitching time was determined by the method described in the earlier paper(1). The time intervals between stages of making from (*a*) adding starter to pitching and (*b*) pitching to hooping are given in Table 1.

All the cheese were ripened under identical conditions. Physical tests and chemical analyses were done on the cheese during ripening and at the end of the experiment. The cheese were then judged independently by four expert cheese-makers (unacquainted with the experiment), by four skilled bakers used to handling a different material but accustomed to make judgements of a somewhat similar nature, and by five non-experts. The experiments were conducted on a small scale and were preliminary in character. For this reason, a full statistical analysis of the results has not been made, the orders in which the cheese were placed by each test or judgement being directly compared. A more complete experiment is being planned, but the results of the first experiment appear to be of sufficient importance to warrant immediate publication.

Physical and chemical tests on the curd and cheese

(The order in which these tests are described corresponds with their position in Table 2.)

(α) *Crumbliness.* At the end of the experiment (cheese 10 weeks old) cylinders taken from the body of the cheese were compressed under loads which increased with the increasing area of the flattening cylinder so that the pressure per unit area remained unchanged. The instrument used (the rheometer) was designed by Dr P. White and Mr J. Cotton and will shortly be described in detail by them. In a series of standard tests on this instrument, the proportion of the cylinders which showed appreciable crumbling during the compression was taken as a criterion of crumbliness.

(β)[1] The phosphate contents of the cheese were determined. Mattick (3) has shown that blue-vein cheese contains a lower quantity of phosphate than hard-pressed cheese and it seemed of interest to find out whether the phosphate content of a hard cheese would be affected by pitching at different consistencies. This might well depend on differences of time in the whey.

(γ)[1] The CaO, which Mattick (3) found to be higher in hard cheese than in blue-vein cheese, was also determined.

(δ)[2] The vapour pressures of the cheese were determined at regular intervals during the ripening process, using the method described by Scott Blair *et al.* (4). (δ) refers to the order at the end of the experiment, the lowest vapour pressure being given first place.

(ϵ) The moisture contents were determined at the same time as the vapour pressures. The ratio vapour pressure/moisture content placed the cheese in the same order as the moisture contents themselves.

(ζ) An apparatus was described in the earlier paper (2) in which a metal sphere is pressed on to the surface of the cheese imitating the pressing with the thumb and fingers of the cheese judge. The cheese are given here in

[1] We are indebted to Dr E. C. V. Mattick and Dr S. J. Rowland for these analyses.

[2] We are indebted to Mr B. Veinoglou for these figures.

order of increasing permanent deformation, i.e. deformations not recovered elastically.

(η) The cheese was listed in order of increasing 'springiness' as determined by the ball compressor, i.e. proportion of total deformation which recovered.

(θ)[1] The chloride contents of the cheese were determined. Since the same amounts of salt were added to all the cheese, differences in chloride content presumably depend on (a) the nature and amount of whey expressed after salting and (b) the subsequent loss in moisture in the cheese. Although, even when great care is exercised, the distribution of salt in cheese may not be very uniform,[2] and although no special precautions were taken in sampling, the results are included because the order correlates better with the order of pitching than with that of moisture contents. We do not, however, wish to draw any conclusions from these data and the whole question is being carefully considered in the planning of further experiments.

(κ)[3] The order of vapour pressures early in the ripening process.

(λ) The order of consistency at pitching point, starting with the firmest curd (longest scald). Note that no. 5 was scalded faster than the rest and no. 4 more slowly.

(μ) Order of titratable acidities when curd was placed on rack.

(ν) The moisture contents of the curd at the pitching point were determined as described in the earlier papers.

(ξ)[4] The titratable acidities were determined at the pitching point.

(π)[4] The pH's were determined at the pitching point.

(ρ) The cheese were placed in order of increasing total (elastic and plastic) deformation as determined by the ball compressor.

(σ) The cheese were placed in order of increasing elastic (recoverable) deformation as determined by the ball compressor.

(τ) The cheese were tested with the spring-skewer described in the earlier paper (2) and placed in decreasing order of force required to cause penetration.

(ϕ) A new method for defining and measuring 'firmness' has been described (5, 6), the data being obtained with the rheometer already mentioned (see α). The cheese was placed in decreasing order of 'firmness' in this sense.

(χ) The tests on (ϕ) were not fully satisfactory in the case of cheese 1. There was insufficient cheese to repeat the test an adequate number of times to place no. 1 with certainty. (χ) thus represents an alternative order to (ϕ).

(ψ) The cheese were placed in order of increasing amount of elastic recovery as determined on the rheometer.

(ω) The cheese were placed in increasing order of 'coefficient of dissipation'. This property is determined on the rheometer and corresponds to the

[1] We are indebted to Dr E. C. V. Mattick and Dr S. J. Rowland for these analyses.

[2] Although McDowall & Dolby (6) have shown that a greater uniformity exists than was previously supposed.

[3] We are indebted to Mr B. Veinoglou for these figures.

[4] We are indebted to Mr H. Hallett for these tests.

extent to which time affects the amount of compression produced when a material is kept loaded under constant stress. Thus a very viscous fluid goes on flowing indefinitely so long as the pressure is maintained (coefficient of dissipation = 1), whereas an elastic solid immediately compresses to its equilibrium strain for the stress applied (coefficient of dissipation = 0). Cheese falls between these two extremes.

Note. In all the above tests the orders were derived from the combined data from an adequate number of replicates.

Judgements on the ripe cheese

The six cheese which had not been used for analysis were subjected to examination by the three types of subjects, i.e. expert cheese-makers (*e*), skilled bakers (*b*) and non-experts (*n*).

In each of three tests (described below) the subjects were asked to judge the firmness of the cheese and to award scores from 1–10, 1 representing the softest imaginable Cheddar cheese, and 10 the firmest. This clearly implied rather a different scaling for the expert cheese-makers from the rest, since they would know much better than the others the possible range of firmness for Cheddar cheese. The result of this was that the cheese-makers tended to give scores covering a smaller range, differentiating by means of halves and even quarters, whereas the bakers and non-experts tended to spread the samples over most of the permitted range of numbers. Since the scores have been used only to derive an order of firmness this difference does not seem important, but it may account for the fact that the cheese-makers tended to give more 'indistinguishable' judgements than did the others.

In the first test, the subjects were told to plunge a skewer of standard pattern into the cheese (a demonstration or explanation was given to the bakers and non-experts). Between tests, the order of the cheese was changed so that the subjects were uninfluenced by any previous test. In the second test, the subjects judged firmness by pressing with the thumbs and fingers on the top of the cheese. In the third test, borings of cheese were taken and handled by the subjects as they pleased.

Initially, the bakers and non-experts were given samples taken by the experimenters with a hollow cylindrical tube, whereas the cheese-makers took their own samples with cheese 'triers'. The experimenter who took the samples (D.V.D.) suspected that samples from some of the cheese were compressed in taking cylinders with the tube borer and, repeating her own tests on the plugs taken with a trier, obtained a substantially different order. Moreover, it had been noticed that at least one of the cheese appeared regularly in quite a different position in the judgement of the cheese-makers than in the judgements of the other categories. The non-experts were therefore asked to repeat the test on samples taken with a trier.

The results of the judgements of each category of subject were pooled and a mean order calculated. The cheese-makers were also asked to judge the

cheese in the normal way for general quality, texture, body, etc. Since these tests were incomplete and only bear indirectly on the problem under discussion, the results are not included as a whole.[1]

Comparison of subjective and objective orders

In order to marshal the sequences obtained from all these tests into some kind of orderly arrangement, slips of paper were taken, each being marked with a code letter (Latin for subjective and Greek for objective tests) and coloured dots were marked on the slips in the mean order for each test and category, a colour being chosen for each of the six cheese. After a few slips had been discarded as of little interest, twenty-one objective and twenty subjective slips remained, the latter including some 'composite' orders (e.g. all the cheese-makers on the skewer test, or all skewer tests irrespective of subject category). The slips were then arranged in two columns in such a way as to bring the most nearly identical orders together both within and between the columns. It is thus possible to see at a glance which categories of subject and of method of subjective testing agree most closely with one another, which physical and chemical tests give the most similar orders and, finally, which subjective tests correspond most closely to any given physical or chemical tests.

These data are given in Table 2, re-lettered to run concurrently. Where several tests seem to fall into a natural group, they are bracketed and lines linking the two sides of the table emphasize similarities which might otherwise be overlooked.

Table 2

Subjective tests			Objective tests	
Subjects' category*	Test	Rank order	Rank order	Test

Discussion

(1) The most striking effect observed in Table 2 is the very close grouping of the objective (physical and chemical) tests around the pitching consistency order, which formed the pivot for the whole experiment. The chemical analyses, perhaps the least significant of these tests, are less closely grouped than the physical tests.

(2) On the subjective side, it is noticeable that the non-experts' orders correspond most nearly to the bulk of the objective orders, then the bakers', the least correlation being shown by the cheese-makers. This does not imply any lack of skill on the part of the latter, since agreement within the group was quite good and it must be remembered that the objective tests were chosen (with a few exceptions) with the express purpose of correlation with firmness and spring. This would suggest that, whereas the non-experts are best able to analyse their sensations and to separate firmness from the other qualities of cheese, the cheese-experts, knowing much more about cheese, found it almost impossible to prevent their judgement of firmness from being influenced by other factors known to be of importance in judging quality.

[1] One of the most highly skilled cheese-makers gave the following order for general quality: 2>1>4>6>3>5.

Table 2

Subjects' category*	Subjective tests: Test		Rank order						Objective tests: Rank order						Test	
n	Boring (cylinder)	*A*	4	5	1	6	2	3	4	1	5	6	2	3	α	Inverse crumbliness
e	Skewer	*B*	4	1	6	2	5	3	4	6 : 1		2 : 5		3	β	Inverse phosphate
n b	Boring (cylinder)	*C*	‡4	1	2	6	5	3	4	1 : 5 : 6			3	2	γ	Inverse calcium oxide
e	Pressing	*D*	4	2	6	1	5	3								
e	Composite†	*E*	§4	2	6	1	5	3								
b	Composite†	*F*	§4	2	6	3	5	1								
b	Skewer	*G*	4	2	6	3	5	1	4	2	3 : 5 : 6			1	δ	Inverse vapour pressure (final)
n b e	Composite†	*H*	‡§4	2	6	3	5	1								
b	Boring (cylinder)	*I*	4	2	1	3 : 6		5	4	2	3	6	1	5	ε	Inverse $\frac{\text{vapour pressure}}{\text{moisture content}}$ and inverse moisture conten
n b e	Boring (cylinder and trier)	*J*	‡§4	2	3	1	6	5								
n	Skewer	*K*	4	6	5	3	2	1	6	4	3	5	2	1	ζ	Ball compressor (inverse plastic deformation)
n	Composite† (excluding trier)	*L*	§4	6	2 : 5		3	1	6	4	5		2 : 3 : 1		η	Ball compressor (% elastic deformation)
n b c	Skewer	*M*	‡4	6	2	5	3	1	6	4	2	5	1	3	θ	Chloride
b	Pressing	*N*	6	2	3 : 4 : 5			1	6	2 : 3 : 4			5	1	κ	Vapour pressure (initial)
									4	6	2	3 : 5		1	λ	PITCHING CONSISTENCY
									4	6	5?	2 : 3		1	μ	Acidity on rack
									4	3 : 6	2	5		1	ν	Inverse moisture at pitch
n	Composite†	*O*	4	3	2 : 6		5	1	4 : 6	3	2	5		1	ξ	Acidity at pitch
									4	3?	6	2	1	5	π	Inverse *p*H at pitch
									6	4 : 3	2	1	5		ρ	Ball compressor (total deformation)
									3	6	2	1	4	5	σ	Ball compressor (inverse elastic deformation)
									6	3 : 2		1 : 4		5	τ	Skewer apparatus
e	Boring (trier)	*P*	2	4	6	3	1	5	2	6	4	3	1	5	φ	'Firmness' (1st order)
n b c	Pressing	*Q*	‡2	4	6	3	5	1	2	6	1	4	3	5	χ	'Firmness' (2nd order)
n	Pressing	*R*	2	4 : 6		3	5	1	2	4 : 6		1	3	5	ψ	Inverse elastic recovery
									4 : 6		2	1	3	5	ω	Inverse coefficient of dissipation
n e	Boring (trier)	*S*	‡2	3	4	1	6	5								
n	Boring (trier)	*T*	3	2	4 : 1		6	5								

* '*n*' indicates non-expert, '*b*' baker, and '*e*' expert cheese-maker.
‡ Composite of different categories of subject.
† Derived from results of all subjective tests.
§ Composite of different tests.

If we were able to award scores to the subjective tests given in the table as follows: $A=1$, $B=2$, $C=3$, etc. (in no sense a score of merit), the mean position for cheese-makers alone would be 7 (G) for b, 9 (I) and for n 13 (M).

(3) The intermediate position of the bakers is most interesting and suggests that the skill of a baker in judging the firmness (body) of dough may be helpful to him in testing cheese. This conclusion must be treated, however, with some reserve until further experiments are completed, since the experimenters formed the opinion that the bakers knew considerably more about cheese than do most non-experts.[1]

(4) It is interesting that apart from crumbliness, which is hardly comparable with the other objective tests, the three sets of data obtained with the rheometer appear together at the bottom of the table.

(5) The pitching order corresponds particularly closely with orders of moisture content, acidity and *p*H taken at pitching and later in the cheese-making process. This is to be expected.

(6) The chloride order corresponds fairly closely, as might be expected, with the (inverse) vapour pressures.

(7) As a preliminary test of the practical utility of the spring skewer, this instrument does not appear in a favourable light. The order does not correspond at all closely with the subjective skewering orders, though agreement with 'firmness' on the rheometer is fairly good. The reason for this is not known.

(8) The ball compressor apparatus fares somewhat better, though the data are hardly adequate to make a fair trial of this instrument. It is hoped to explore this question more fully in the near future.

(9) The inverted order of coefficients of dissipation (ω) is difficult to place. The position in Table 2 corresponds best with neighbouring objective tests but is, nevertheless, more closely related on the subjective side to I and J. The physical significance of this property will be discussed elsewhere.

(10) Non-experts judging borings taken with the cylinder were apparently mainly influenced by crumbliness, whereas on samples taken with the trier, a very different order was obtained. This suggests that, in future experiments, great care must be taken to ensure that all borings are taken with the same instrument and, as nearly as possible, in the same way.

(11) The effect of a more rapid scald (cheese 5) is most marked. The cheese becomes softer than the corresponding no. 3 and is much more 'springy', thus tending to approximate more to the body of Dutch cheese. Cheese 4 was scalded more slowly than the others, but being pitched firmest anyhow, it was not possible to distinguish the two effects.

(12) In conclusion, we would point out both the limitations and advantages of this method of presenting data. The mere ranking of samples in order

[1] One of the bakers was blind. His orders do not differ markedly from the rest, though he showed perhaps some tendency to give more 'indistinguishable' judgements. The data available for a single subject was, however, inadequate for us to draw any significant conclusions on this interesting point.

neglects differences in spacing, which are sometimes important. Thus, no distinction is made between barely significant and very marked differences. On the other hand, when numbers of data are limited, the method has the advantage of giving a ready survey of results where a more detailed statistical analysis is not justified. It may be recalled that the chance of a complete agreement between two orders of six cheese occurring purely by coincidence is only one in 720. This figure will give some idea of the probable significance of the data given in Table 2, though it must be borne in mind that the tests and categories are by no means independent of one another, many of the properties measured being controlled by the same factors. Where a set of data consists of a sum of two or more other series already given, this fact has been clearly indicated in the table and should be borne in mind.

Summary

1. Six cheese of Cheddar type were made under conditions as similar as possible, except that the consistency of the curd at pitching, and in two cases the rate of scald, were varied.

2. Chemical and rheological analyses, as well as measurements of moisture content and vapour pressure, were made on the cheese and also judgements of firmness under various conditions were given by cheese-makers, bakers and non-experts.

3. A table is given in which the orders of placing of the cheese are compared as between the objective tests and the subjective judgements. The cheese-makers and the non-expert testers gave, in general, characteristically different orders, the orders given by the bakers tending to fall between the two.

4. It appears that the consistency of the curd at the pitching point (W/h as determined by the Scott Blair-Coppen method) and probably the rate of scald, are prime factors in determining the firmness of the cheese.

5. The whole experiment is preliminary in character and it is intended to repeat it in an extended form in the near future.

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637.143

283. THE KEEPING QUALITY OF MILK POWDERS

PART I. ADDITION OF ANTIOXIDANTS

By R. WAITE
The Hannah Dairy Research Institute, Kirkhill, Ayr

In the last 10–15 years increasing attention has been focused on a group of substances, roughly classified as 'antioxidants', which have a marked influence on the rate of oxidation of unsaturated animal and vegetable oils and fats. In this group are included relatively simple, well-known compounds such as hydroquinone, pyrogallol and pyrocatechol, together with lesser known complexes whose structure appears to be closely allied to that of vitamin E(1). The function of the antioxidant seems to be that of extending the induction period, during which oxygen uptake by the fat is slow, thus delaying the onset of the second stage, marked by a rapid rise in rate of oxidation, which is usually associated with the development of unpleasant 'tallowy' flavours in the fat. It has been suggested(2) that antioxidants delay fat oxidation by inhibiting the formation of the initial moloxides which are the precursors of peroxide formation, but that they cause no change in the system after the end of the induction period. From the work of Mattill(3) it appeared that the antioxygenic capacity of many substances lay in the possession of a hydroxy substituted benzene nucleus and that the most effective form was one in which the hydroxyl group was attached in the *o*- or *p*-positions, or preferably in both. As antioxidants for a lard-cod-liver oil mixture, α-naphthol was shown to be roughly twenty times and hydroquinone about eighty times more powerful than β-naphthol. Many substances, some non-aromatic, such as maleic and malonic acids(4) have been found to possess antioxidative properties in various degrees, whilst amongst the more complex antioxidants come the fractions containing the unsaponificable lipoids of the cereal germ oils(5) and also several cereal flours(6).

The need for some method of delaying the onset of tallowiness in whole milk powders, particularly spray dried, has always been evident and the addition of substances capable of this to reinforce the antioxidants normally present in milk appeared to offer a simpler approach to this problem than elaborate gas-packing precautions. The twin considerations of the legality and palatability of the added substance considerably reduce the number of possible antioxidants and unfortunately exclude hydroquinone, which appears to be the most effective so far recorded for animal fats. In these circumstances it was natural to turn first to those foodstuffs or their fractions, such as the cereal flours, which had been found to possess antioxidative capacity.

EXPERIMENTAL

As a preliminary trial in a series planned to cover a large number of naturally occurring antioxidants, it was decided to investigate the possibility of using additions of oat flour as a method of delaying tallowiness in spray-dried whole-milk powder (as the type of milk powder most susceptible to oxidation).

The oat flour was added in four different ways, using three concentrations and a control powder in each series. These were as follows:

(1) 0·1, 0·25 and 0·5 % oat flour (by weight) added to the milk before condensing;

(2) 0·1, 0·25 and 0·5 % oat flour (by weight) added to the milk after condensing;

(3) 0·1 and 0·5 % oat flour (on the weight of raw milk) gelatinized and added to the condensed milk;

(4) 1·0, 2·5 and 5·0 % oat flour added to a commercial spray-dried powder; and also

(5) 0·5 % hydroquinone, calculated on the weight of butter-fat, added to the milk (*a*) before, and (*b*) after condensing.

The reason for the inclusion of series (2) was that, as the oat flour used gelatinized at about 63° C., the condensing temperature of 60° C. (25″ vac.) (chosen to avoid lipase activity) might cause some proportion of the oat flour to gelatinize and it was not known whether this would affect the antioxidative activity. Series (5) was introduced to give some idea of the amount of protection that might be afforded by a strong antioxidant, against which the efficacy of the oat flour might be assessed.

Prior to condensing, which was carried out in a glass 5 l. flask, the butter-fat concentration of each batch of raw milk was adjusted to 3·3 %. The powder was produced on a laboratory spray-drying machine(7) from the milk of a single Ayrshire cow, two-thirds through her fourth lactation, fed on a normal production ration which did not vary over the period of the experiment.

The powder of each series was divided and stored in small amber glass bottles at 37° C., each bottle containing just sufficient for one determination of solubility, peroxide value and taste. One bottle from each series was opened at fortnightly intervals over a period of 16 weeks, so that in this way the original oxygen-to-powder ratio was the same for all samples.

The results are given in Tables 1 and 2. The following comments may be made.

Solubility

The solubility index of the powders was determined by the method of Howat *et al.*(8) at each examination. No significant change occurred over the whole period except that the introduction of the highest concentration of the relatively insoluble oat flour caused a slight irregularity in the individual values. The moisture content of all samples lay between 2·0 and 3·2 %.

Taste and peroxide value

The tasting test was conducted as follows: 8 g. of powder were reconstituted in 64 ml. of water at room temperature and the taste assessed by five tasters, none of whom knew the identity of the numbered samples. In order to avoid contaminating the palate with the tallowy flavour, the samples were first placed in an agreed order of smell, the tallowy ones being tasted last.

The control powders showed a considerably greater resistance to oxidation than previous spray-dried whole-milk powders stored at 37° C. and this tended to minimize the effect of the oat flour additions. The exact cause of this increased keeping quality is not known, but utilization of clean, winter milk some 4–5 hr. after milking, in glass and stainless steel apparatus with subsequent storage in non-metallic containers probably contributed to it. The taste of oat flour in aqueous concentrations of about 0·1 % is, unfortunately, not unlike the 'cardboardiness' associated with the first stages of fat oxidation and the agreement between members of the tasting panel during the first weeks of the storage period was less than in any previous tests carried out in this laboratory.

As noted by previous workers (6) it was found that up to a concentration of 0·25 % in the reconstituted milk the oat flavour was not noticeable but that it could be readily distinguished at 0·5 %, giving a characteristic, rather pleasant taste to the milk. Gelatinization of the flour prior to adding it to the milk appeared to bring out the oat flavour in the early stages of the storage period with complete loss in the concluding weeks. Added directly to the manufactured powder, the oat-flour flavour was rather more apparent at the lower concentration of 0·25 % than in the other samples. The powder containing hydroquinone was very unpalatable from the beginning, a somewhat 'metallic' flavour being immediately noticed in the reconstituted milk, developing into an unpleasant aftertaste; storage caused no further development of this defect.

It will be seen from Table 1 that an addition of 0·1 % oat flour was insufficient to cause any appreciable delay in the development of talloviness, but that 0·25 % oat flour added to the milk before condensing, or 2·5 % added to the dry powder, delayed actual talloviness (but not 'cardboardiness') by about 4 weeks. On the basis of the known temperature coefficient of fat oxidation, this would be equivalent to about 4 months at ordinary temperatures. Addition of 0·5 % oat flour prevented tallowiness in all samples over the entire storage period except when gelatinized before addition to the milk.

The peroxide figures did not reach very high values in any sample but corresponded quite well with the results of the tasting tests.

It may be remarked that, of the oat-flour series, only the control samples would have been definitely inedible at the end of the 16-weeks storage at 37° C.

Table 1. *Peroxide values* (9) *and tasting tests*

% oat	(1) Added before condensing	(2) Added after condensing	(3) Gelatinized before addition	(4) Added dry to powder*

Table 1. *Peroxide values* (9) *and tasting tests*

% oat flour added to raw milk	Time stored at 37°C. (weeks)	(1) Added before condensing		(2) Added after condensing		(3) Gelatinized before addition		(4) Added dry to powder*	
		Peroxide value ml. $N/200$ $Na_2S_2O_3$	Taste	Peroxide value ml. $N/200$ $Na_2S_2O_3$	Taste	Peroxide value ml. $N/200$ $Na_2S_2O_3$	Taste	Peroxide value ml. $N/200$ $Na_2S_2O_3$	Taste
0	0	Nil	Good	Nil	Good	Nil	Good	Nil	Good
	2	,,	Slt. cardboardy	,,	,,	,,	Slt. cardboardy	,,	,,
	4	,,	,,	,,	Slt. cardboardy	,,	,,	,,	Slt. cardboardy
	6	,,	,,	,,	,,	,,	,,	,,	,,
	8	,,	,,	,,	,,	,,	,,	,,	,,
	12	0·20	Slt. tallowy	,,	Slt. tallowy	0·20	Slt. tallowy	0·40	Slt. tallowy
	14	0·80	,,	1·20	,,	0·80	,,	0·40	,,
	16	1·92	Tallowy	1·40	Tallowy	1·92	Tallowy	0·60	,,
0·10	0	Nil	Good	Nil	Good	Nil	Good	Nil	Good
	2	,,	,,	,,	,,	,,	,,	,,	,,
	4	,,	,,	,,	Slt. cardboardy	,,	Slt. cardboardy	,,	Slt. cardboardy
	6	,,	,,	,,	,,	,,	,,	,,	,,
	8	,,	Slt. cardboardy	,,	Cardboardy	,,	Slt. cardboardy and cooked	,,	,,
	12	,,	,,	,,	Very slt. tallowy	1·40	Tallowy and slt. cooked	0·20	,,
	14	0·48	Very slt. tallowy	1·40	Slt. tallowy	2·20	,,	0·20	,,
	16	1·32	Slt. tallowy	0·80	,,	1·80	,,	0·40	,,
0·25	0	Nil	Good	Nil	Good	—	—	Nil	Good, oatmeal flavour
	2	,,	,,	,,	,,	—	—	,,	,,
	4	,,	Slt. cardboardy	,,	Slt. cardboardy	—	—	,,	,,
	6	,,	,,	,,	,,	—	—	,,	Slt. cardboardy
	8	,,	Cardboardy	,,	,,	—	—	,,	,,
	12	,,	,,	,,	Cardboardy	—	—	0·20	,,
	14	0·20	,,	0·50	Slt. tallowy	—	—	0·20	,,
	16	0·60	Very slt. tallowy	0·60	,,	—	—	0·30	,,
0·50	0	Nil	Good	Nil	Good	Nil	Good, oatmeal flavour	Nil	Good, oatmeal flavour
	2	,,	,,	,,	,,	,,	,,	,,	,,
	4	,,	,,	,,	Good, slt. oatmeal flavour	,,	Slt. cardboardy, less oat flavour	,,	,,
	6	,,	Slt. cardboardy or oatmealy	,,	,,	,,	,,	,,	,,
	8	,,	,,	,,	Good, oatmeal flavour	,,	Slt. tallowy, no oat flavour	,,	,,
	12	,,	,,	,,	,,	1·20	,,	0·48	Good, very slt. cardboardy, oat flavour
	14	0·20	Oat flour distinguishable	0·60	,,	0·60	,,	0·20	,,
	16	0·48	Fairly good, oat flavour distinguishable	0·40	,,	1·00	,,	0·40	,,

* The percentage oat flour added to the powder was 1·0, 2·5 and 5·0.

As the literature had suggested, hydroquinone proved (Table 2) a very much more effective antioxidant than oat flour, no tallowiness or appreciable peroxide value developing even at the end of 4 months, whether added to the milk before or after condensing. Since the 'metallic' taste due to the hydroquinone differed markedly from the 'cardboardy' or tallowy tastes produced by fat oxidation, it is unlikely that the latter were masked by the former, a conclusion borne out by the very low peroxide values.

Table 2. *Series* 5. *Addition of* 0·5 % *hydroquinone on the weight of fat present in the raw milk*

Hydroquinone added	Time of storage at 37° C. (weeks)	Peroxide value ml. *N*/200 $Na_2S_2O_3$	Taste
None	0	Nil	Good
	2	,,	Slt. cardboardy
	4	,,	,,
	6	,,	,,
	8	,,	,,
	12	0·20	Slt. tallowy
	14	0·80	,,
	16	1·92	Tallowy
To milk before condensing	0	Nil	Foreign, 'metallic' flavour leaving an unpleasant, persistent aftertaste. No tallowiness was noted in any sample. No real difference between samples to which the hydroquinone had been added to the milk before or after condensing
	2	,,	
	4	,,	
	6	,,	
	8	,,	
	12	,,	
	14	0·20	
	16	0·10	
To milk after condensing	0	Nil	
	2	,,	
	4	,,	
	6	,,	
	8	,,	
	12	,,	
	14	0·10	
	16	0·10	

Discussion

In view of the results obtained with hydroquinone it seems clear that, provided a suitable antioxidant could be found, the general principle of adding antioxidants as a means of retarding butter-fat deterioration during the storage of whole-milk powders is sound. It is true that the results obtained with oat flour are not so striking, and that too high a concentration of this substance adversely affects the taste of the milk powder, but these preliminary trials appear sufficiently conclusive to warrant further investigation of other naturally occurring antioxidants. In this connexion there is no doubt that cereal germ oils offer promising possibilities and the present work is being extended to include these and other active preparations.

In the experiments reported above it is evident that the amount of hydroquinone used was unnecessarily high. Presumably such a reagent, even if it had no deleterious effect on palatability, would not be legally permissible as

an addition to milk powder. It would nevertheless be of interest to determine the minimum quantity necessary to prevent tallowiness in spray-dried whole-milk powder over an indefinite period *so that such an addition could provide a base-line* against which to measure the efficacy of less powerful antioxidants. This matter is receiving attention in further trials now in progress. In a preliminary survey such as this the use of an accelerating temperature for storage seemed justified, but in future work it will be necessary to ensure that high storage temperatures do not themselves introduce anomalous effects by causing premature breakdown of the antioxidant (e.g. ascorbic acid) and a range of storage temperatures, including normal room temperature, will be used.

On the other hand, the addition of pro-oxidants such as copper to catalyse the rate of fat oxidation would be of doubtful value, since this would introduce an unknown factor (the copper-antioxidant reactivity) and might thus mask the direct effect of the antioxidant on the butter-fat.

SUMMARY

1. Hydroquinone is an effective antioxidant for the butter-fat of spray-dried whole-milk powder, but in a concentration equivalent to 0·5 % of the weight of fat (0·12 % on the weight of powder) it produces an objectionable 'metallic' flavour in the powder and reconstituted milk.

2. Oat flour is much less efficient than hydroquinone as an antioxidant but 0·25 % added to the milk, preferably before condensing, increases the resistance of the resultant powder to the development of tallowiness by the equivalent of about 4 months at normal temperatures. Raising the concentration of oat flour to 0·5 % approximately doubles this increase but at the same time imparts a noticeable oat flavour to the powder.

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637.143 + 637.344

284. THE EFFECTS OF ADDITIONS OF DRIED SKIM MILK AND DRIED WHEY ON THE BAKING QUALITY AND NUTRITIVE PROPERTIES OF WHITE BREAD

By K. M. HENRY, J. HOUSTON, S. K. KON and J. POWELL
From the National Institute for Research in Dairying, University of Reading
and R. H. CARTER and P. HALTON
From the Research Association of British Flour-Millers, St Albans

(With 3 Figures)

Introduction

Even in peace time it is not easy to compound an optimal diet without the use of milk or milk products. With the simplified and restricted diets which war conditions impose on belligerent nations this is an admitted impossibility. This paramount importance of milk in war-time nutrition makes it imperative to utilize it as fully as possible and to safeguard for human consumption those milk products or by-products which are either largely wasted in peace time or are normally used for feeding livestock. Skim milk and whey are two such products. Skim milk, on account of its high content of good quality protein, calcium and other salts, of riboflavin and other water-soluble vitamins, is an excellent protective food and an ideal supplement to the principal sources of energy, cereals and potatoes. The same, though to a lesser extent, can be said of whey. Both can be economically used for human consumption only after the removal of water. One of the easiest ways of including them in the diet is by adding them to bread. Much bread in Canada and in the U.S.A. has been made for some years from a mix to which dried separated milk powder has been added. Some of the nutritional effects of adding milk to bread have recently been studied in America by Fairbanks (1,2), who has also reviewed in detail the earlier literature on the subject. To his list the three papers by Hale (3), Hoffman (4) and Babecki (5) may be added. The bread studied by the two former contained large quantities of whole milk and other additions and was a special 'fancy' bread.

In this country milk and milk products have been used in breadmaking to only a very small extent. The present joint investigation was undertaken to study the technical and nutritional aspects of such additions.

When considering the use of dried milk products in breadmaking it is necessary to take into account not only their influence on the nutritive value but also their effect on the various processes involved in the manufacture of bread and on the appearance and palatability of the finished article. The importance of this has been stressed by Skovholt & Bailey (6).

According to the method of manufacture the addition of dried milk to dough may degrade or improve the bread. Greenbank *et al.*(7) obtained bread of larger volume and better texture with milk which, before drying, had been preheated to a high temperature. Milk preheated to 75–95° C. gave the best results. On the other hand milk which had been preheated lightly or not at all lowered the volume and quality of the bread. Though the preheating of condensed milk before drying increases its viscosity, Johnson & Ward(8) have found that milks of high viscosity are not necessarily the best for breadmaking. Allen & Bell(9) have suggested that the beneficial effect of preheating is due to the coagulation of lactalbumin which otherwise has a detrimental effect on the gluten.

Another factor influencing the quality of the bread made with the addition of dried milk is the character of the flour used. The reason for such differences between flours is not clear(7,10,11).

There are comparatively few references in the literature to the use of dried whey in breadmaking. Fisher(12) noted that the incorporation of 5–10 % made the doughs considerably more sticky. The crust of the bread was somewhat biscuit-like in character and the crumb was more open in grain. Allen & Bell(9) found that liquid whey depressed the volume increase of the dough during fermentation to a greater extent than liquid milk but that on boiling this depressing effect was reversed in both cases. This would suggest that, as with dried milk, the method of preparation of dried whey may be an important factor in determining its suitability for use in breadmaking.

EXPERIMENTAL

I. *The effect of dried milk products on the baking quality of bread*

The milk and whey used in the experiments described in this paper were roller-dried products supplied by the Milk Marketing Board as typical commercial samples. Physical tests in the laboratory followed by practical tests in the bakehouse were made to determine to what extent the addition of the dried milk or dried whey altered the water-absorbing power of flours. These tests showed that when 6 % of the flour was replaced by 6 % of the milk powder the absorption of the mixture was 1 gallon per sack of 280 lb. greater than the absorption of the flour alone. The addition of 6 % of the whey powder had the reverse effect and lowered the absorption by 1 gallon per sack. When the quantity of water used was adjusted accordingly, doughs were obtained which handled satisfactorily. As fermentation progressed the extra water in the milk doughs minimized the tightening or binding which had been noticed by previous workers and with the whey doughs the use of less water minimized the excessive stickiness noticed by Fisher(12). The milk and whey doughs were of the same general consistency as the control doughs containing no addition, but were somewhat less elastic and had a slight tacky or sticky feel.

The effect of increasing amounts of skim milk powder on loaf volume is shown by the data in Table 1. The two flours A and B were commercial bread-making flours obtained from different mills and differed in that the former was treated with improvers while the other was untreated.

The bread was made on a short straight dough system using 2 % yeast and $1\frac{1}{4}$ % salt with a total fermentation time of $3\frac{1}{4}$ hr. to the oven.

Of the loaves made from flour A, that containing 2 % milk was rather smaller than the control and had a rather tougher crumb and a deeper cream colour. The change brought about by the addition of 4 % milk powder was much greater. The crumb of the loaf was more rubbery than that of the 2 % milk loaf, the grain closer and the colour deeper. Increasing the milk content to 6 or 8 % was accompanied by progressive changes in the loaf characters, although the change from 4 to 6 or 6 to 8 % was less than from 2 to 4 %. With this flour increasing the milk-powder content from 8 to 10 % brought about a big drop in loaf volume.

Table 1. *Effect of skim milk powder on loaf volume*

% milk powder	Loaf volume (ml.)		Decrease in volume (%)	
	Flour A	Flour B	Flour A	Flour B
0	3380	2830	—	—
2	3190	2715	6	4
4	2890	2330	14	16
6	2750	2280	18	18
8	2640	2200	22	20
10	2280	2150	32	22

The bread from flour B was loaf for loaf much poorer than that from flour A, but showed the same changes with increasing milk content. Thus the addition of 2 % milk was accompanied by a small decrease in volume and a slight toughening of the crumb, while the addition of 4 % milk brought about a much more marked change in these characteristics.

Further experiments confirmed that the use of 2 % of this particular milk powder was accompanied by a deterioration in bread quality small enough to be ignored. The use of 4 % or more materially altered the bread.

The milk powder had a marked influence on the colour of both crust and crumb. The lactose in the milk is not fermented by yeast and is caramelized in the oven. As a result the crust of the milk bread has a rich golden brown colour. This increased colour is very marked when as much as 6 % of milk is used.

The colour of the crumb became more creamy with 2 % milk but as this creamy colour had a reddish tinge it assumed a brownish shade with increased milk content.

When considering the flavour of bread regard has to be paid to the fact that it is eaten with bacon, meat and fish as well as by itself or with sweetened products such as jam. For this reason bread should have a neutral flavour. The addition of milk produces a rather sweeter and richer flavour which is

pleasant when bread is eaten by itself or with jam but which does not blend so well with salty foods such as bacon. In our experiments small quantities up to 2 % changed the flavour comparatively little, but the change with 6 % milk was sufficiently pronounced to be noticed when the bread was eaten with meat. Such products would be classed as 'fancy bread'.

In Table 2 data are given showing the effect on loaf volume of the addition of dried whey to two flours C and D.

Flour C was untreated, while flour D, which was obtained from the same firm as flour A, had been treated with improvers. As a result the control bread from D represented both in volume and other characters the optimum that the flour would give, whereas the control loaf from C could have been improved by suitable treatment of the flour.

The addition of 1 and 2 % of whey to flour C resulted in a small increase in volume, 3 and 4 % had no appreciable effect, a 5 % addition resulted in a small fall in volume and with 6 % whey a marked diminution occurred. As

Table 2. *Effect of dried whey on loaf volume*

% dried whey	Loaf volume (ml.)		Change in volume (%)	
	Flour C	Flour D	Flour C	Flour D
0	2920	3540	—	—
1	2980	3360	+ 2	− 5
2	3060	3140	+ 5	−11
3	2890	3200	− 1	−10
4	2920	3060	0	−14
5	2840	2970	− 3	−16
6	2630	2850	−10	−19

far as the internal characteristics of the bread are concerned no appreciable change occurred up to 5 % of whey, which resulted in a slight toughening of the crumb. This became more pronounced with 6 % whey.

The addition of increasing quantities of whey to flour D resulted in a progressive decrease in loaf volume. This was accompanied by a progressive slight toughening of the crumb. The fall in loaf volume was not accompanied by a corresponding and proportional deterioration in general quality, which remained good.

The addition of increasing amounts of whey also produced a progressive browning of the outside of the loaf and of the crumb. This was just noticeable with 1 % whey and was quite marked with 6 %.

The flavour of the bread was also affected, a definite cheesy taste being noticed, particularly at the higher concentrations of 5 and 6 %. In previous baking trials with dried whey it had been noticed that the intensity of this flavour varies from sample to sample and increases as the whey becomes older. Experience has shown that whether this flavour is liked or disliked is a matter of taste, but in any case unless the amount used is kept down to about 2 % there is a definite change from the neutral flavour of water bread.

To study the effects of milk and whey additions on the nutritive value of bread a series of experimental breads was prepared according to formulae given in Table 3. Both substances were added at a 2 % and also at a 6 % level. The analyses of the dough ingredients are given in Table 4.

In addition to the trials with the samples of dried milk and whey already described further tests were made with a sample of spray-dried skim milk obtained from another source. The incorporation of this product in bread also lowered its quality, although to a less extent than did a corresponding amount of the roller-dried sample. In view of the results obtained by other workers

Table 3. *Dough formulae of the breads used in the various experiments*

Type of bread	Flour (g.)	Tap water (ml.)	Yeast (g.)	Salt (g.)	Dried milk (g.)	Dried whey (g.)	$CaHPO_4$ (g.)	$CaCO_3$ (g.)	Casein (g.)
Control	800	426	16	10	—	—	—	—	—
2 % milk	784	426	16	10	16	—	—	—	—
6 % milk	752	454·5	16	10	48	—	—	—	—
2 % whey	784	426	16	10	—	16	—	—	—
6 % whey	752	398	16	10	—	48	—	—	—
Control + $CaHPO_4$	798	426	16	10	—	—	2·127	—	—
2 % milk + $CaHPO_4$	783	426	16	10	16	—	1·414	—	—
2 % whey + $CaHPO_4$	782	426	16	10	—	16	1·760	—	—
6 % whey + $CaHPO_4$	751	398	16	10	—	48	1·035	—	—
Control + $CaCO_3$	798	426	16	10	—	—	—	1·5773	—
Control + casein	800	468	16	10	—	—	—	—	17·400
Control + $CaCO_3$ + casein	800	472	16	10	—	—	—	1·4424	17·360
6 % whey + $CaCO_3$ + casein	800	427	16	10	—	48	—	0·6732	10·944

Table 4. *Analyses of the dough ingredients (moisture-free basis)*

Ingredient	% moisture	% Ca	% P		% N	μg. vit. B_1/100 g.	μg. riboflavin/100 g.
			Total	Phytin			
Flour	14·43	0·016	0·114	0·042	2·29	75·7	30·6
Yeast	73·70	0·023	1·840	—	9·16	2020·0	929·1
Dried milk	7·26	1·417	1·052	—	5·90	360·7	1657·6
Dried whey	6·24	0·718	0·770	—	2·10	416·4	1518·5
$CaHPO_4$	—	29·73	—	—	—	—	—
$CaCO_3$	—	40·09	—	—	—	—	—
Casein (Glaxo, flavin-free)	2·27	—	—	—	15·39	—	—
Tap water	—	0·0126	—	—	—	—	—

such variations are to be expected and it is clear that the effects on bread quality resulting from the addition of dried milk can only be considered in relation to the particular sample used. Further, while the sample of roller-dried skim milk could not be used in greater amount than 2 % without causing a definite deterioration in bread quality, some modification of the method of manufacture, such as preheating the liquid milk before drying, should yield a product that could be incorporated in bread in greater amounts. It is also probable that as far as its effects on breadmaking quality are concerned a better type of dried whey could also be manufactured. The manufacture on a commercial scale of dried milk and dried whey suitable for baking purposes might well lead to the greater use of these products in the baking industry.

II. *Effects of dried milk products on the nutritive value of bread*

The most conspicuous results of the addition of dried milk and whey to bread are increases in the calcium, phosphorus, nitrogen and vitamin content of the loaf. The nutritional effects of these changes were measured by biological tests on rats, which included metabolic studies and also by chemical estimations of vitamin B_1 and riboflavin.

The breads were baked at St Albans from flour A (Table 1) three times a week and were despatched the following day. They were stored in bread bins and used for 2 or 3 days.

For the metabolic experiments, however, the breads were sliced and dried in a hot-air tunnel at 140° F., and were then milled. A single large batch of each bread, prepared for this purpose, lasted throughout the experiments.

1. *Balance experiments.*

(A) *Calcium and phosphorus.* A deficiency of calcium is one of the most important nutritional shortcomings of white bread. It contains much more phosphorus (Ca:P ratio about 1:5) but over one-third of this is phytin phosphorus of low availability (Table 4). Dried skim milk and, to a lesser extent, dried whey add to bread a relatively large quantity of calcium and also of phosphorus in a form which is completely available. The economy of calcium is closely bound up with that of phosphorus, and it is clear that the value of bread-milk or bread-whey combinations cannot be judged on analytical figures but must be assessed in metabolic experiments. The planning and general technique of these experiments were as in our earlier work (13). The breads were fed as an exclusive diet but were supplemented with certain vitamins. Vitamin D was not added.

For technical reasons it was possible to study simultaneously the retentions of Ca and P from only four of the breads. For this purpose the unsupplemented white bread (control), 2 and 6 % milk and 6 % whey breads were used.

Five litters of 22–25 day old male rats weighing 41–66 g. were allocated by lot to the four breads. The intake of the breads was equalized between litter-mates. For feeding, the breads were mixed with 2·5 times their weight of distilled water. The animals received daily—separately from the diet—4μg. β carotene, 15μg. aneurin and 30μg. riboflavin, and were given distilled water to drink. The experiment lasted for 5 weeks. At the end of the experiment the rats were killed by coal gas, the femora and humeri were removed and their ash content determined as described by Kon & Henry (14).

Results. The results of this experiment are shown in Tables 5–7. Table 5 gives the analyses of the breads. Table 6 shows individual and mean figures for the intakes, excretions and retentions of Ca and P. Table 7 gives the statistical analyses of some of the data, for which the *t*-test of 'Student' as described by White (15) was used.

During the course of the experiment two rats, no. 4 on 6 % whey and no. 10 on 2 % milk breads, did not clear up their allowance of food for several days. As this reduced unreasonably the food intake of their litter-mates these two animals were dropped from the experiment on the 27th and 22nd day respectively, but continued to receive the diet. Both died from pneumonia on the 33rd day of the experiment.

It is evident from Table 5 that the effect of the milk and whey additions on the calcium content of bread is very marked. Calcium is more than doubled with 2 % milk and more than quadrupled with 6 %. In this respect 6 % whey was slightly superior to 2 % milk. Both these additions brought the Ca content of white bread up to or above that of wholemeal bread (16). Owing to the higher phosphorus content of bread the changes in this element are not so conspicuous. The Ca/P ratio shows a marked improvement from 0·2 for the control bread to 0·63 for the 6 % milk loaf. Judging by the standards of Sherman & Macleod (17) and Sherman & Quinn (18) the supply of calcium from all breads except 6 % milk was well below the normal requirements, while all breads supplied more than enough phosphorus even if allowance is made for phytin

Table 5. *Ca, P and moisture contents of the experimental breads*

Bread	% Ca	% P	Ca/P	% moisture
Control	0·0244	0·1207	0·20	5·94
2 % milk	0·0552	0·1465	0·39	3·39
6 % milk	0·1112	0·1778	0·63	4·38
6 % whey	0·0662	0·1592	0·42	5·43

phosphorus which is only partly available and which constituted nearly a third of the phosphorus of the flour (Tables 4, 6).

Table 6 shows that the absolute retention of Ca increased with the dietary level. The percentage retention of calcium on the unsupplemented bread was only 60 % though the element was in short supply and there was a relative excess of phosphorus. We have previously shown (19) that under optimal conditions in the presence of vitamin D the unavoidable loss of calcium by the rat amounted to some 0·2 mg. daily in the urine and 0·1 mg. in the faeces when milk supplied the calcium, and to 0·2 and 0·4 mg. respectively when the calcium was derived from calcium acid phosphate. The latter figures are the same as those obtained now. This indicates that on such low intake, retention could not be higher than 60 %, and that the calcium of white bread is as available as that of $CaHPO_4$, but slightly less available than that of milk.

A comparison of the flour and water analyses in Table 4 with those of breads in Tables 5 and 12 shows that in hard water districts a large part of the bread calcium is derived from tap water.

The addition of 2 % milk or 6 % whey raised the percentage retention to about 80 %, and this was not bettered by 6 % milk. Table 7 shows that this improvement is statistically highly significant, but that, as would be expected, the slight differences between the supplemented breads are without significance.

Table 6. *Individual and mean figures for the intakes, excretions and retentions of calcium and phosphorus*

Table 6. *Individual and mean figures for the intakes, excretions and retentions of calcium and phosphorus*

Rat no.	Wt. of rat (g.) Initial	Final	Bread intake (g.)	Ca intake (g.)	Ca excretion (g.) Urine	Faeces	Ca retention g.	%	P intake (g.)	P excretion (g.) Urine	Faeces	P retention g.	%	mg. Ca intake/ g. gain wt.	mg. P intake/ g. gain wt.	% bone ash
							Control bread									
1	56	71	210·39	0·0513	0·0078	0·0101	0·0334	65·10	0·2539	0·1369	0·0553	0·0617	24·31	3·4	16·9	41·5
5	66	91	222·08	0·0542	0·0063	0·0089	0·0390	71·96	0·2681	0·1352	0·0473	0·0856	31·93	2·2	10·7	41·3
9	52	81	229·69	0·0560	0·0045	0·0151	0·0364	65·00	0·2772	0·1225	0·0634	0·0913	32·94	1·9	9·6	39·0
13	45	76	223·65	0·0546	0·0102	0·0104	0·0340	62·27	0·2699	0·1148	0·0574	0·0977	36·19	1·8	8·7	34·8
17	46	65	193·79	0·0473	0·0077	0·0204	0·0192	40·59	0·2339	0·1196	0·0529	0·0614	26·25	2·5	12·3	38·9
Mean (5 rats)	53·0	76·8	215·92	0·0527	0·0073	0·0130	0·0324	60·98	0·2606	0·1258	0·0553	0·0795	30·32	2·4	11·6	39·1
							2 % milk bread									
2	52	71	208·53	0·1150	0·0056	0·0182	0·0912	79·30	0·2933	0·1378	0·0664	0·0891	30·38	6·1	15·4	44·4
6	63	91	222·83	0·1229	0·0077	0·0088	0·1064	86·56	0·3134	0·1484	0·0414	0·1236	39·44	4·4	11·2	44·0
10*	53	49	162·85	0·0898	0·0054	0·0103	0·0741	82·50	0·2290	0·1337	0·0475	0·0478	20·87	—	—	43·7
14	44	76	224·72	0·1239	0·0108	0·0151	0·0980	79·09	0·3161	0·1237	0·0648	0·1276	40·37	3·9	9·9	42·0
18	42	63	191·57	0·1057	0·0098	0·0197	0·0762	72·11	0·2694	0·1141	0·0605	0·0948	35·18	5·0	12·8	43·3
Mean (4 rats)	50·3	75·3	211·91	0·1169	0·0085	0·0154	0·0930	79·27	0·2981	0·1310	0·0583	0·1088	36·34	4·9	12·3	43·4
							6 % milk bread									
3	58	74	210·98	0·2346	0·0090	0·0449	0·1807	77·02	0·3751	0·1517	0·0991	0·1243	33·14	14·7	23·4	49·6
7	62	90	222·98	0·2480	0·0102	0·0310	0·2068	83·39	0·3965	0·1455	0·0741	0·1769	44·62	8·9	14·2	48·4
11	57	91	229·94	0·2557	0·0078	0·0288	0·2191	85·69	0·4088	0·1380	0·0695	0·2013	49·22	7·5	12·0	51·7
15	45	80	224·66	0·2498	0·0122	0·0325	0·2051	82·12	0·3994	0·1375	0·1014	0·1605	40·19	7·1	11·4	46·6
19	45	67	193·97	0·2157	0·0119	0·0531	0·1507	69·87	0·3449	0·1227	0·0861	0·1361	39·46	9·8	15·7	47·4
Mean (5 rats)	53·4	80·4	216·51	0·2408	0·0102	0·0381	0·1925	79·62	0·3850	0·1391	0·0860	0·1598	41·33	9·6	15·3	48·7
							6 % whey bread									
4*	51	56	173·25	0·1146	0·0063	0·0141	0·0942	82·21	0·2758	0·1455	0·0605	0·0698	25·31	22·9	55·2	43·9
8	61	81	222·81	0·1474	0·0078	0·0101	0·1295	87·86	0·3547	0·1796	0·0581	0·1170	32·98	7·4	17·7	45·2
12	55	86	226·76	0·1500	0·0099	0·0111	0·1290	86·00	0·3610	0·1535	0·0702	0·1373	38·04	4·8	11·6	47·5
16	46	75	223·69	0·1480	0·0102	0·0200	0·1178	79·60	0·3561	0·1447	0·0692	0·1422	39·94	5·1	12·3	42·7
20	38	60	192·48	0·1273	0·0149	0·0283	0·0841	66·07	0·3064	0·1491	0·0588	0·0985	32·15	5·8	13·9	46·0
Mean (4 rats)	50·0	75·5	216·44	0·1432	0·0107	0·0174	0·1151	79·88	0·3446	0·1567	0·0641	0·1238	35·78	5·8	13·6	45·4

* Died on the 33rd day of the experiment, data omitted from the means.

A simple calculation shows that about 95 % of the calcium added to bread by 2 % milk and about 93 % of the calcium supplied by 6 % whey were retained by the rat. These values are very close to the values observed by us previously for milk (19).

One might have expected that more than 80 % calcium would be retained from the 6 % milk bread. Here, however, the calcium intake was probably already slightly in excess of the requirements of the animals at their slow rate of growth (17). This view is strengthened by the marked increase observed in their faecal excretion of calcium (Table 6, cf. Henry & Kon (19)).

Table 7. *Statistical analysis of some of the data in Table 6*

Dietary difference tested	Control–2 % milk (4 rats)	Control–6 % milk (5 rats)	Control–6 % whey (4 rats)	2 % milk–6 % milk (4 rats)	2 % milk–6 % whey (3 rats)	6 % milk–6 % whey (4 rats)
			% Ca retention			
Difference	59·98–79·27 = −19·29	60·98–79·62 = −18·64	59·96–79·88 = −19·92	79·27–78·10 = +1·17	79·25–77·84 = +1·41	80·27–79·88 = +0·39
S.E.M.	±3·41	±3·29	±2·14	±1·41	±2·33	±1·83
*P**	1:93 Significant	1:217 Significant	1:357 Significant	1:2 Not significant	1:1 Not significant	1:1 Not significant
			% P retention			
Difference	29·67–36·34 = −6·67	30·32–41·33 = −11·01	31·83–35·78 = −3·95	36·34–39·35 = −3·01	38·33–35·02 = +3·31	43·37–35·78 = +7·59
S.E.M.	±1·01	±2·11	±1·06	±1·18	±1·74	±2·63
*P**	1:135 Significant	1:152 Significant	1:30 Significant	1:12 Not significant	1:5 Not significant	1:16 Not significant
			% bone ash			
Difference	39·1–43·4 = −4·3	39·1–48·7 = −9·6	38·5–45·4 = −6·9	43·4–48·0 = −4·6	43·1–44·6 = −1·5	48·5–45·4 = +3·1
S.E.M.	±1·04	±1·10	±1·03	±0·23	±0·60	±0·63
*P**	1:39 Significant	1:1,190 Significant	1:135 Significant	Over 1:11,111 Significant	1:8 Not significant	1:62 Significant

* P = Probability that a mean difference at least as great as the observed mean difference would have arisen by random sampling from a homogeneous population.

The absolute retention of phosphorus rose steadily with increasing level and so did the percentage absorption, except that this was slightly lower from the 6 % whey bread than from the 2 % milk loaf.

Percentage retentions from all three supplemented breads were significantly better than from the control (Table 7), but the differences between them, though suggestive, did not reach the conventional level of statistical significance, most probably owing to the small number of animals in any one group. Even the control bread had enough phosphorus to cover normal requirements (18). It did not, however, contain enough calcium to support efficient utilization. The improvement in phosphorus retention which followed the addition of milk or whey was most probably due to the extra calcium they supplied.

The bones of the animals receiving the control bread were poorly calcified and the ash content of the bones was progressively improved by the addition

of 2 % milk, 6 % whey and 6 % milk. The improvement over the control group was in all cases statistically significant. The difference between 2 % milk and 6 % whey was not significant, but 6 % milk was statistically superior to both.

The addition of 6 % milk solids to bread was followed, as can be seen from Table 6, by more economical gains in weight as rats receiving this bread invariably gained more in weight than their litter-mates which received the same quantities of control bread. On the other hand the addition of 2 % milk or of 6 % whey had no such effect.

(B) *The biological value and digestibility of the proteins.* It is well known that the proteins of milk can supplement and enhance the value of the proteins of cereal foods (20,21,22). For this reason a metabolic study of the biological value of the bread-milk and bread-whey combination was carried out. Most of the results reported in the literature were obtained on mixtures containing some 20–25 % of milk proteins. In the breads used by us milk supplied only

Table 8. *Composition and analysis of diets used in the nitrogen metabolism experiment*

Type of bread	Control	2 % milk	6 % milk	2 % whey	6 % whey
Bread	63·3	58·5	56·2	64·0	64·3
Margarine fat	10·0	10·0	10·0	10·0	10·0
Salta*	4·0	4·0	4·0	4·0	4·0
Potato starch	10·0	10·0	10·0	10·0	10·0
Rice starch	12·7	17·5	19·8	12·0	11·7
% N, exp. I	1·415	1·391	1·432	—	—
% N, exp. II	1·374	—	—	1·350	1·377
% moisture, exp. I	7·68	6·27	7·49	—	—
% moisture, exp. II	7·75	—	—	7·51	8·11

* de Loureiro (1931). *Arch. Patol. Lisboa*, **3**, 72.

about 5 and 15 % of the protein according to level, while whey contributed even less, under 2 and under 6 % respectively. It was of interest to see whether supplementary effects would become manifest at these lower levels.

The biological values and true digestibilities of the five types of bread were determined in two separate experiments by the method of Mitchell (21,23). In the first the control bread was compared with the two milk breads and in the second with the two whey breads.

The breads were fed at an 8·5 % level of protein (N × 6·25) intake. Table 8 gives the composition and analyses of the experimental diets which were fed mixed with 1½ times their weight of distilled water. The cages, vitamin additions, methods of collection and analysis of excreta and the composition of the standardizing low egg-nitrogen diet have been described elsewhere by Henry *et al.* (24). In both experiments four groups of three litter-mate female rats were used, the breads were tested in turn on each rat by alternating the diets in each period. In the first experiment, the animals were 22–25 days old and weighed 41–58 g. at the start of the experiment. For the whey-bread comparison the animals were 24–25 days old and weighed 37–56 g.

Results. The results are given in Tables 9, 10 and 11. Table 9 shows the individual nitrogen metabolism data for the milk experiment (*a*) and the whey experiment (*b*). Table 10 gives mean figures for the biological values and true digestibilities of the nitrogen of the different breads and Table 11 gives the statistical analysis of the results. In the whey experiment rat no. 13 died of pneumonia during the second experimental period; consequently, results are only available for eleven rats.

It will be seen from Table 10 that the addition of both 2 and 6 % milk to bread caused an improvement in the biological value of the proteins which was statistically significant for the higher level of milk (Table 11).

The addition of either level of whey to bread had no effect on its biological value.

The addition of milk or of whey to the bread caused a slight lowering of the true digestibility. This effect was more marked in the case of whey. For both substances the lowering was progressive and was statistically significant for the two whey breads but only for the higher level milk bread (Table 11).

The increase in the biological value of the nitrogen of white bread caused by the addition of milk was only slight. The data at our disposal do not allow us to decide with certainty whether this increase was simply the expected weighted mean of the biological values of the two components or whether it denotes a true supplementary relationship. To demonstrate such a relationship it is necessary to measure not only the biological value of the proteins of the mixture of foods but also that of the two separate components. Of these three necessary values our experiments have only yielded two, nor could a reliable estimate of the third, the biological value of the milk nitrogen, be easily obtained. The reason is that the process of baking causes some injury to the proteins, at any rate to those in the bread crust (25, 26). While the extent of this loss is probably immaterial for a comparison of two types of bread, it is difficult to assess for the milk component. We have therefore not measured separately the biological value of the nitrogen of the dry skim milk used by us. For the purpose of an indirect comparison we may well use the figure given by Fairbanks & Mitchell (27) for choice commercial roller-process powder. If it is assumed that the value, 81·6, was not altered by baking, a calculation based on data from Tables 8 and 10 gives an expected weighted mean value for the 2 % milk bread of 47·6 and for the 6 % milk bread of 49·7. The values actually found were 46·5 and 49·8 respectively, showing no supplementary relationship between milk and bread proteins at those low levels of milk admixture. If the value for 'slightly scorched' roller-dried skim milk (27) is taken, the figures 45·8 for 2 % milk and 47·9 for the 6 % milk bread are obtained. Even these, however, indicate only a very slight supplementary relationship. This result is at first sight surprising in view of the well-known ability of the milk proteins to supplement those of cereal grains. The explanation probably lies in the relatively low levels at which milk was added to breads in the present experiment. A similar observation has been reported by Mitchell & Hamilton (28),

Rat no.

1 2 3 4 5 6 7 8 9 10 11 12

1 2 3 4 5 6 7 8 9 10 11 12

1 2 3 4 5 6 7 8 9 10 11 12

1 2 3 4 5 6 7 8 9 10 11 12

1 2 3 4 5 6 7 8 9 10 11 12

Table 9. *Individual nitrogen metabolism data*

(*a*) Milk-bread combinations

Rat no.	Bread tested	Weight of rat (g.) Initial	Final	Diet intake (g.)	N intake (mg.)	Faecal urinary N (mg.)	N (mg.)	Biological value	True digestibility
				Low egg-nitrogen diet					
1	—	55	59	43·00	—	89·7	66·7	—	—
2	—	51	54	41·48	—	84·4	66·2	—	—
3	—	56	58	42·98	—	88·2	68·1	—	—
4	—	56	58	36·50	—	67·8	75·0	—	—
5	—	58	60	36·50	—	77·9	73·3	—	—
6	—	55	57	36·47	—	67·1	73·4	—	—
7	—	44	50	40·50	—	71·6	76·7	—	—
8	—	43	47	37·75	—	68·1	68·0	—	—
9	—	46	50	40·07	—	85·4	72·8	—	—
10	—	45	49	32·50	—	58·6	76·3	—	—
11	—	42	44	31·40	—	69·3	61·0	—	—
12	—	42	44	27·34	—	59·9	79·1	—	—
				First experimental period					
1	Control	61	64	39·00	551·8	133·5	336·7	46·2	90·4
2	2 % milk	57	61	37·44	520·8	135·4	295·2	50·9	89·1
3	6 % milk	61	63	38·97	558·0	159·6	298·5	52·0	85·4
4	Control	61	62	34·50	488·1	93·7	333·6	43·9	94·5
5	2 % milk	61	61	34·32	477·4	107·5	282·2	52·1	92·7
6	6 % milk	59	60	34·20	489·7	103·6	309·1	47·0	92·0
7	Control	52	54	35·00	495·2	114·7	335·7	42·5	90·6
8	2 % milk	48	50	32·10	446·5	114·7	286·8	44·0	87·9
9	6 % milk	53	54	34·98	500·8	130·0	292·0	51·1	89·1
10	Control	51	53	31·64	447·7	89·5	324·3	40·4	94·2
11	2 % milk	46	48	33·24	462·3	107·6	268·5	51·6	92·4
12	6 % milk	46	50	33·50	479·6	107·6	305·9	49·3	93·9
				Second experimental period					
1	6 % milk	69	71	42·00	601·3	158·6	339·6	50·0	89·5
2	Control	64	67	40·77	576·9	148·9	336·8	48·0	89·6
3	2 % milk	71	71	41·97	583·7	151·4	336·8	49·0	88·1
4	6 % milk	67	73	42·00	601·3	123·9	355·3	50·4	93·4
5	Control	66	69	39·05	552·5	120·3	370·1	42·1	93·0
6	2 % milk	64	71	41·33	574·9	138·7	345·3	46·7	89·7
7	6 % milk	59	60	38·50	551·3	152·0	317·1	50·3	87·2
8	Control	55	58	38·10	539·1	145·4	344·5	41·5	87·0
9	2 % milk	58	58	34·61	481·4	147·5	326·7	38·8	87·0
10	6 % milk	56	58	32·04	458·6	139·4	272·9	50·3	85·0
11	Control	53	56	34·23	484·3	130·0	307·2	43·7	88·3
12	2 % milk	54	58	36·00	500·7	124·2	316·6	48·1	91·5
				Third experimental period					
1	2 % milk	74	76	40·50	563·3	159·0	333·3	45·7	86·2
2	6 % milk	70	74	40·48	579·7	166·0	314·8	50·8	87·1
3	Control	69	76	40·02	566·2	139·6	302·6	54·0	88·8
4	2 % milk	79	81	42·49	591·0	130·5	374·1	46·2	92·8
5	6 % milk	75	79	42·06	602·2	137·8	374·1	45·8	91·6
6	Control	76	80	41·78	591·0	136·3	367·8	44·6	90·9
7	2 % milk	65	66	40·50	563·3	147·0	344·3	47·2	90·4
8	6 % milk	61	64	40·46	579·3	162·0	325·8	48·7	86·4
9	Control	64	65	38·84	549·5	134·7	363·4	42·5	93·0
10	2 % milk	60	64	38·54	536·2	161·6	307·5	51·1	87·2
11	6 % milk	60	64	39·98	572·5	165·1	312·2	50·4	85·9
12	Control	62	64	39·99	565·8	148·5	345·7	46·9	90·1
				Low egg-nitrogen diet					
1	—	83	91	50·50	—	100·4	73·3	—	—
2	—	79	83	47·78	—	110·8	66·2	—	—
3	—	79	85	50·50	—	93·5	74·3	—	—
4	—	85	89	44·00	—	94·4	76·7	—	—
5	—	81	86	43·99	—	90·0	77·5	—	—
6	—	84	90	46·05	—	92·7	66·8	—	—
7	—	70	76	46·44	—	114·2	71·5	—	—
8	—	67	75	47·84	—	103·0	67·5	—	—
9	—	68	76	48·37	—	108·6	66·0	—	—
10	—	64	64	31·62	—	82·8	74·0	—	—
11	—	67	71	39·50	—	82·4	68·8	—	—
12	—	64	67	37·32	—	87·8	64·0	—	—

Table 9 (*continued*)

(*b*) Whey-bread combinations

Rat no.	Bread tested	Weight of rat (g.) Initial	Weight of rat (g.) Final	Diet intake (g.)	N intake (mg.)	Faecal urinary N (mg.)	Faecal urinary N (mg.)	Biological value	True digestibility
				Low egg-nitrogen diet					
14	—	55	58	41·81	—	82·4	71·9	—	—
15	—	57	64	50·50	—	81·6	76·5	—	—
16	—	52	56	43·87	—	83·6	60·0	—	—
17	—	48	55	47·30	—	81·7	58·9	—	—
18	—	53	60	48·26	—	79·1	61·3	—	—
19	—	49	54	45·35	—	84·3	61·0	—	—
20	—	49	53	44·22	—	85·5	62·0	—	—
21	—	51	56	46·78	—	93·0	59·8	—	—
22	—	46	48	36·21	—	87·6	*57·6	—	—
23	—	46	49	40·43	—	80·9	60·8	—	—
24	—	39	42	36·06	—	79·9	52·2	—	—
				First experimental period					
14	2 % whey	62	61	30·96	417·8	86·4	288·5	45·4	94·4
15	6 % whey	67	65	33·00	454·4	116·2	298·2	44·0	86·8
16	Control	58	60	34·14	469·1	95·8	284·1	49·4	93·7
17	2 % whey	57	58	36·00	486·0	114·0	312·1	42·6	89·8
18	6 % whey	62	64	36·00	495·8	119·4	281·6	50·1	88·4
19	Control	54	56	26·62	365·8	82·7	231·9	48·5	91·2
20	2 % whey	55	56	29·76	401·8	89·4	274·3	43·2	92·9
21	6 % whey	58	59	30·16	415·5	102·9	252·2	49·2	90·3
22	Control	51	54	27·88	383·1	78·7	242·9	51·0	97·3
23	2 % whey	52	56	30·88	416·9	95·5	257·0	49·8	92·6
24	6 % whey	44	46	27·02	372·1	85·8	203·7	56·9	93·3
				Second experimental period					
14	Control	64	66	34·32	471·7	80·1	331·6	43·9	98·2
15	2 % whey	67	72	38·50	519·8	127·4	341·0	42·7	88·8
16	6 % whey	62	66	34·02	468·6	118·5	288·0	46·0	89·0
17	Control	58	64	38·46	528·4	112·6	314·5	47·9	92·3
18	2 % whey	66	70	38·46	519·2	123·9	301·1	48·4	89·3
19	6 % whey	58	62	33·97	467·8	128·4	273·3	47·5	86·7
20	Control	58	64	37·38	513·6	116·5	307·0	48·7	92·9
21	2 % whey	61	67	37·46	505·8	124·3	300·2	48·7	91·4
22	6 % whey	55	62	36·82	507·0	124·6	287·2	52·4	93·6
23	Control	57	61	36·06	495·5	121·4	298·5	48·1	91·4
24	2 % whey	49	53	30·97	418·1	108·0	262·2	46·4	91·3
				Third experimental period					
14	6 % whey	67	71	34·99	481·8	109·0	329·6	40·3	90·1
15	Control	75	79	40·81	560·8	128·1	373·2	42·0	91·0
16	2 % whey	66	70	35·87	484·3	112·7	328·2	46·7	91·2
17	6 % whey	67	72	43·97	605·6	165·8	356·6	44·3	86·7
18	Control	74	79	41·48	569·9	130·3	355·0	44·1	90·6
19	2 % whey	63	67	33·92	458·0	127·5	287·4	42·8	86·9
20	6 % whey	65	71	39·73	547·1	158·3	323·7	45·4	87·4
21	Control	69	73	36·27	498·3	137·4	305·3	45·7	88·7
22	2 % whey	63	67	35·67	481·5	123·8	296·4	47·7	93·1
23	6 % whey	62	65	35·23	485·2	148·9	290·4	45·3	85·8
24	Control	54	56	30·94	425·2	97·3	250·9	51·9	94·3
				Low egg-nitrogen diet					
14	—	76	80	42·36	—	93·5	73·1	—	—
15	—	83	98	66·48	—	132·1	84·2	—	—
16	—	79	91	59·03	—	119·9	71·9	—	—
17	—	78	88	58·65	—	124·4	70·0	—	—
18	—	85	100	63·86	—	122·9	73·4	—	—
19	—	72	85	56·66	—	114·7	66·5	—	—
20	—	75	88	57·61	—	135·5	67·1	—	—
21	—	75	88	53·97	—	125·0	70·4	—	—
22	—	70	75	42·31	—	108·8	63·2	—	—
23	—	68	76	45·70	—	108·1	63·7	—	—
24	—	59	64	37·98	—	91·9	60·5	—	—

who, on recalculating Hart & Steenbock's(29) data for the value for pigs of maize-milk combinations, found little or no supplementary effect when milk supplied from 3 to 20 % of the total proteins but a marked one with 28·5 %. It might also be recalled that Fairbanks(2) noticed in paired feeding experiments no effect on the protein content of rats of the addition of 6 and 12 % milk solids to bread. On the other hand in experiments which have not yet been published we found a marked supplementary relation between the proteins of white bread and those of cheese when the latter supplied 50 % of the total proteins.

Table 10. *Mean biological values and true digestibilities of the nitrogen of the different breads*

Bread	Control	2 % milk	6 % milk	Control	2 % whey	6 % whey
			Biological value			
Period I	43·2	49·7	49·9	49·6	45·3	50·1
Period II	43·8	45·7	50·3	47·1	46·5	48·6
Period III	47·0	47·5	48·9	45·9	45·8	43·8
Mean	44·7	47·6	49·7	47·4	45·9	47·4
			True digestibility			
Period I	92·4	90·5	90·1	94·1	92·4	89·7
Period II	89·5	89·1	88·8	93·7	90·2	89·7
Period III	90·7	89·2	87·8	91·1	90·4	87·5
Mean	90·9	89·6	88·9	92·9	91·1	88·9

Table 11. *Statistical analysis of the data in Table* 10

Difference tested	Control–2 % milk	Control–6 % milk	2 % milk–6 % milk	Control–2 % whey	Control–6 % whey	2 % whey–6 % whey
			Biological value			
Difference	−2·9	−5·0	−2·1	+1·5	0·0	−1·5
S.E.M.	±1·41	±0·97	±1·29	±1·15	±1·11	±1·31
*P**	1:16 Not significant	1:5000 Significant	1:7 Not significant	1:4 Not significant	1:1 Not significant	1:4 Not significant
			True digestibility			
Difference	+1·3	+2·0	+0·7	+1·81	+4·0	+2·2
S.E.M.	±0·90	±0·67	±0·84	±0·70	±0·79	±0·79
*P**	1:5 Not significant	1:76 Significant	1:1 Not significant	1:40 Significant	1:1667 Significant	1:47 Significant

* See footnote to Table 7.

The mean biological value, 46, obtained by us at an 8·5 % level of protein intake for the nitrogen of white bread[1] may be compared with the value of 52 found by Mitchell & Carman(21) and of 47 obtained by Klein *et al.*(30) for patent white flour, in both cases at an 8 % level. Much higher values, 74 for young animals and 83 for mature rats, were reported by French & Mattill(31) for the proteins of white bread fed at a 5 % level of protein intake. The lower level of protein feeding used by the latter authors may account for the discrepancy. The interpretation of their results is, however, complicated by the fact that their white bread contained an unstated quantity of milk.

[1] In recent unpublished experiments we obtained under similar conditions a value of 48.

The effects of the addition of milk solids to bread cannot be judged solely by the improvement in the biological value of the protein, since the protein content is also affected. A calculation of the 'protein value' which takes this as well as the digestibility into account (32) yields, on the dry basis, a figure of 4·4 for unsupplemented white bread and of 5·4 for the 6 % milk loaf, an increase of 25 %.

2. *The vitamin B_1 and riboflavin content of the breads.*

Both vitamins were measured by fluorimetric methods, details of which have been given in full elsewhere (33,34).

(A) *Vitamin B_1*. For the estimation of vitamin B_1 20 g. of flour, 10 g. of yeast or 20 g. of dried finely ground bread were suspended in 75 ml. of water at *p*H 2 and incubated for 24 hr. at 37° C. with 0·1 g. of Parke, Davis and Co. pepsin. A further 0·1 g. of pepsin was then added and incubation continued for another 24 hr. The *p*H was then adjusted to 4 and the digest incubated for 6 hr. with takaphosphatase (240 mg. takadiastase: Parke, Davis and Co.). Further treatment was as already described (33). Five samples of each type of bread were analysed in the course of the first experiment on the feeding of the breads as an exclusive diet (p. 199). The results are given in Table 12. One sample of each of the three breads used in the second experiment (p. 203) and of the flour and yeast from which they were made, and two samples of each of the breads fed in the third experiment (p. 207), were also analysed, as well as the milk and whey used in baking (Table 4).

It will be seen that ordinary white bread contains about 1μg. of vitamin B_1 per g. of dry matter. This is in agreement with the estimate of Drummond & Moran (35). The increase on addition of milk or whey was very much the same, the 2 % milk or whey loaf containing some 1·1μg./g. dry matter and the 6 % loaves 1·3μg./g. dry matter, an increase of some 30 % over white bread. It will be noted that a rat consuming the equivalent of 6 g. of dry white bread daily will obtain from it 6μg. of vitamin B_1, a quantity probably sufficient for satisfactory growth (36,37).

(B) *Riboflavin*. 20 g. of dried bread or flour or 10 g. of yeast were extracted for ½ hr. under reflux four times with 75% aqueous methanol. The extracts were combined and the alcohol removed in vacuo. The aqueous residue was brought to *p*H 4 with glacial acetic acid and treated with 0·5 ml. of a 4% solution of $KMnO_4$ the excess of which was then removed by 1 ml. of 3% H_2O_2. After adjusting the *p*H to 3, 0·5 g. of fuller's earth (B.D.H.) were added. After shaking for 1 hr. the fuller's earth was removed by centrifuging and the fuller's earth process was repeated twice more. The combined adsorbates were extracted five times with 10 ml. lots of a mixture of 20 ml. redistilled pyridine, 80 ml. of water and 2 ml. glacial acetic acid. The combined extracts were made up to 50 ml. Of this, half was oxidized again with 0·5 ml. 4% $KMnO_4$ and 1 ml. of 3% H_2O_2 and half was reduced with 1 g. of solid $Na_2S_2O_4$ to give the 'blank' reading. Both solutions were examined fluorimetrically and the riboflavin content of the sample was obtained by subtracting the 'blank'.

The riboflavin content of the dried milk and whey was measured by the method already described (34). The estimations were carried out on the substances measured for vitamin B_1 and the results are given in Tables 4 and 12. It is possible that the recovery of riboflavin from bread by the method used by us was not complete. In fact, values calculated for the 6 % milk bread

from the figures for the riboflavin content of the dried milk which we know to be correct (38) are definitely higher than those given by the direct assay (142μg./100 g. against 80–100μg./100 g.). It is therefore probable that the contribution of milk to the riboflavin content of bread is even greater than appears from our figures. Taken at their face value our figures for the riboflavin content of white bread show that a rat consuming daily the equivalent of 6 g. of dry bread would obtain only 2·2μg. of riboflavin, an amount insufficient for satisfactory growth (38). It would seem therefore that in white bread riboflavin rather than vitamin B_1 is a limiting factor.

Table 12. *Analyses of the crumb of the various breads fed as an exclusive diet (moisture-free basis)*

Bread	% moisture*	% Ca	% P	Ca/P	% N	μg. vit. B_1/100 g.	μg. riboflavin/100 g.
			First experiment				
Control	44·42	0·030	0·123	0·24	2·29	99·5	36·7
2 % milk	44·21	0·057	0·138	0·41	2·30	106·1	44·0
6 % milk	44·62	0·120	0·182	0·66	2·48	135·3	86·4
2 % whey	44·36	0·045	0·133	0·34	2·25	108·9	49·0
6 % whey	44·01	0·075	0·166	0·45	2·24	133·3	96·9
Control + $CaHPO_4$	44·66	0·117	—	—	—	—	—
2 % milk + $CaHPO_4$	44·69	0·116	—	—	—	—	—
6 % milk	45·36	0·117	—	—	—	—	—
2 % whey + $CaHPO_4$	44·42	0·115	—	—	—	—	—
6 % whey + $CaHPO_4$	43·96	0·113	—	—	—	—	—
			Second experiment				
Control	44·69	0·027	—	—	—	86·5	37·2
Control + $CaCO_3$	44·39	0·120	—	—	—	86·5	33·4
6 % milk	44·85	0·117	—	—	2·74	110·3	81·3
Control + $CaCO_3$ + casein	44·01	0·119	—	—	2·86	—	—
			Third experiment				
Control + $CaCO_3$ + casein	44·34	0·104	—	—	2·63	94·7	35·7
6 % whey + $CaCO_3$ + casein	43·53	0·098	—	—	2·49	119·2	97·1
6 % milk	44·38	0·100	—	—	2·53	120·3	102·0

* Mean of daily estimations made during the experiment.

3. *The effect of skim milk and whey additions on the value of white bread as an exclusive diet.*

The experiments so far described have dealt with improvements in separate nutrients resulting from the addition of skim milk or whey to white flour. To study the more general effects of such additions on the total nutritive value of white bread we have carried out several experiments in which the breads were fed to rats as the sole diet. As the experiments progressed and nutritive defects became obvious various additions were made to some or all of the breads.

(A) *First experiment.* Seven groups of five newly weaned litter-mate male rats weighing 34–50 g. were placed in individual cages on screens. They were allocated by lot to the five experimental breads. Only the crumb of the fresh breads was fed. The breads were offered ad libitum and a record of intake was kept for each rat in the course of the first 8 weeks of the experiment. Uneaten

bread and scattered pieces were collected each morning. These leavings were dried two or three times a week and their equivalent in fresh bread calculated from the moisture figures given in Table 12. Daily estimations were made of the moisture content of the bread. These varied so little from day to day that the average figure was used for the calculation of total intake at the end of the experiment. As white flour contains very little provitamin A(39) a daily supplement of 4 i.u. of β carotene was given to all rats from the seventh week of the experiment. The results are shown in Fig. 1 and Table 13. It will be seen from the figure that right from the start and throughout the first 8 weeks of the experiment the rats receiving the 6 % milk bread showed the best and those receiving the unsupplemented bread the poorest growth. The rats

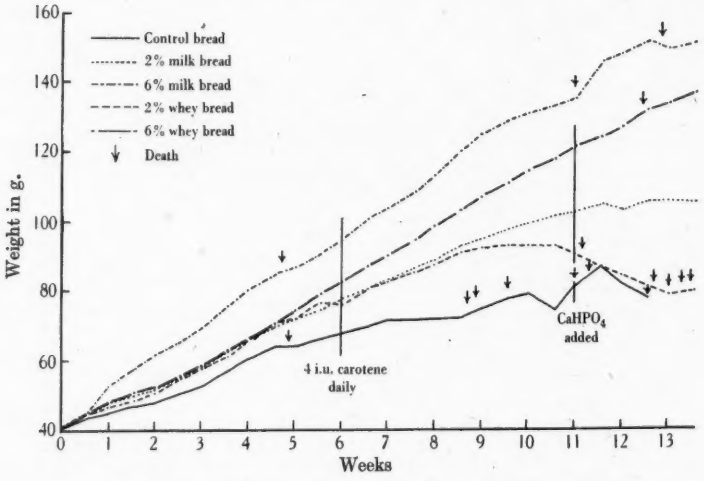

Fig. 1. Growth of rats receiving various breads as an exclusive diet. First experiment.

receiving the remaining three breads grew for the first 5 weeks at very much the same rate, but after that the animals getting the 6 % whey bread definitely drew ahead. Differences in growth were accompanied by differences in food intake (Table 13), but the efficiency of utilization of food increased as whey and milk were added. It is of interest to note that though the intakes of the 2 and 6 % whey breads were very similar, the gains in weight and hence the efficiency of utilization were decidedly higher for the second of these breads. Our results may be compared with those of Fairbanks(1), who obtained during the first 8 weeks of his experiment gains per g. of dry intake of 0·077 g. on unsupplemented white bread and 0·167 g. on 6 % milk bread. Our values were respectively 0·101 g. and 0·186 g. The addition of milk increased the efficiency of utilization of food in the first instance in the ratio of 2·2:1 and in the second of 1·9:1.

The analytical figures given in Table 12 show how deficient white bread is in calcium and how unfavourable is its Ca/P ratio. One of the most striking

Table 13. *Performance, during the first* 8 *weeks, of rats receiving various experimental breads as an exclusive diet.* (*First experiment*)

Rat no.	Weight of rat (g.) Initial	Gain	Bread intake (g.)	g. gain/g. bread intake	% bone ash (at death)
		Control bread			
9945*	37	21	467	0·045	32·4
9985†	42	27	509	0·053	34·1
9991*	41	36	562	0·064	29·6
9996†	50	47	662	0·071	33·6
10027	38	Dead	—	—	—
10036*	43	25	525	0·048	29·9
10055*	36	28	557	0·050	28·9
Mean	41·5	30·7	547·0	0·055	31·42
		2 % milk bread			
9949	37	43	595	0·072	39·3
9982	41	45	593	0·076	34·3
9989	41	50	619	0·081	37·0
9998	45	54	651	0·083	41·1
10030	43	56	668	0·084	39·0
10038	42	48	587	0·082	39·1
10058	37	40	562	0·071	41·7
Mean	40·9	48·0	610·7	0·078	38·79
		6 % milk bread			
9944	39	58	637	0·091	47·4
9981	45	63	616	0·102	45·9
9992†	38	47	608	0·077	44·6
9995	46	Dead	—	—	—
10028†	41	100	880	0·114	48·0
10037	42	91	793	0·115	45·4
10057	34	84	767	0·110	49·4
Mean	39·8	73·8	716·8	0·102	46·78
		2 % whey bread			
9947†	35	41	576	0·071	35·7
9979†	42	43	601	0·072	33·5
9993†	38	43	622	0·069	32·7
9997†	46	59	704	0·084	34·5
10029†	46	50	721	0·069	35·3
10035	45	44	616	0·071	34·6
10056	36	48	579	0·083	36·0
Mean	41·1	46·9	631·3	0·074	34·61
		6 % whey bread			
9946	43	49	581	0·084	43·0
9980	41	62	656	0·095	43·8
9990†	40	61	653	0·093	37·7
9999	45	64	719	0·089	43·7
10026	40	62	649	0·096	46·3
10033	40	58	625	0·093	43·5
10054	36	51	590	0·086	42·6
Mean	40·7	58·2	639·0	0·091	42·94

* Subsequently died before $CaHPO_4$ was added to the breads.
† Subsequently died after $CaHPO_4$ was added to the breads.

effects of the addition of 6 % milk is the quadrupling of the calcium content of the bread and the accompanying marked and satisfactory change in the

Ca/P ratio. For this reason it seemed to us at first that the main benefit derived from the addition of milk or whey lay in the additional calcium they supplied, and we originally planned to change the diet after 8 weeks by bringing the calcium level in all breads, by the addition of $CaHPO_4$, to that in the 6 % milk loaf. Owing to transport difficulties the change was delayed for 3 weeks, during which the original diet was continued but the bread intake was not measured. By that time five of the rats receiving unsupplemented white bread had died, the rats on the 2 % whey bread were losing weight and those getting the 2 % milk bread were almost stationary. Actually this addition of calcium had very little effect either on the growth rate of the animals or in preventing further deaths. It may have come too late to counteract the effects of prolonged calcium deficiency, the results of which could be clearly seen in the bones of the rats, which either died during or were killed at the end of the experiment. The ash content of the bones increased progressively with the addition to bread of 2 % whey, 2 % milk, 6 % whey and 6 % milk, i.e. in the order of increasing calcium content, but did not reach normality (Table 13). The severity of the obvious skeletal changes, such as spontaneous rib fractures and beading, blurring and angulations of the costochondral junction, followed a similar trend, and it is of interest that shaft fractures were noticed even in the ribs of most rats receiving the 6 % milk bread but that the costochondral junction appeared in them macroscopically normal. The pathological changes in the ribs of the rats receiving the other breads were much more severe. Multiple fractures, angulations, and even collapses of the thoracic wall, were observed in most of them.[1] From the data of Tables 12 and 13 it is possible to calculate the calcium intake of the rats consuming the different breads and to see to what extent their calcium requirements were satisfied during the first 8 weeks of the experiment. The amounts derived from the breads are set out in Table 14. Our rats were given tap water to drink. If the assumption of Bing & Mendel(40) is correct they would take about 1·3 times as much water as dried food, i.e. the rats eating white bread would take $304 \times 1{\cdot}3 = 395$ ml. Of this 243 ml. were present in the fresh bread and the animals presumably drank 152 ml. Our tap water contains 0·0425 g. Ca per l. This would add 6·4 mg. Ca to their intake. The column giving the total Ca intake per g. gain shows that even when this allowance is made the calcium intake of all groups was well below the optimum requirements as defined by Sherman & Macleod(17).

This shortage of calcium in the diet, the relatively high level of phosphorus and the lack of vitamin D created conditions conducive not only to the development of rickets but also of tetany(41). Typical tetanic seizures were, in fact, seen in one rat receiving the ordinary bread and in one rat receiving the 6 % milk bread. All animals surviving at the end of the experiment were

[1] It should be remembered that calcium phosphate was added towards the end of the experiment to all but the 6 % milk bread and that this may have affected the ash content of the bones of some of the animals (cf. Table 13).

killed and the following figures for serum calcium were obtained on pooled blood in each group (Table 15).

There were a number of deaths in the course of the experiment, most numerous in the control and 2 % whey groups. All rats in the former and all but two in the latter died before the experiment was completed. In all cases the lungs were involved, probably as an effect of the marked skeletal changes.

Three rats died in the 6 % milk group. In two of them a twist of the bowel was present and in the third pneumonia and septicaemia were the cause of death. Finally, twist of the bowel was again responsible for the only death in the 6 % whey bread group.

Table 14. *Data on gains in weight and Ca intakes of the rats receiving the various experimental breads in the three experiments*

Type of bread	Bread intake dry basis (g.)*	Calculated intake of tap water (ml.)†	Ca intake (mg.) Bread	Tap water	Total	Gain in weight (g.)	mg. Ca intake/g. gain weight
			First experiment				
Control	304	152	91·2	6·4	97·6	30·7	3·2
2 % milk	341	173	201·2	7·4	208·6	48·0	4·3
6 % milk	397	196	480·4	8·3	488·7	73·8	6·6
2 % whey	351	176	158·0	7·5	165·5	46·9	3·5
6 % whey	358	184	268·5	7·8	276·3	58·2	4·7
			Second experiment				
Control + $CaCO_3$	777	390	932·8	16·6	949·4	101·9	9·3
6 % milk	837	407	979·2	17·3	996·5	131·5	7·6
			Third experiment				
Control‡	195	—	202·8	—	202·8	43·5	4·7
6 % whey‡	206	—	201·9	—	201·9	45·4	4·4
6 % milk	205	—	205·0	—	205·0	46·8	4·4

* The consumption of fresh bread is given in Tables 13, 16 and 17. † Cf. p. 202.

‡ + $CaCO_3$ + casein.

Table 15. *Blood serum calcium of rats receiving various breads*

Bread	No. of rats	mg. Ca/100 ml. serum
2 % milk	7	7·15
6 % milk	4	6·51
6 % whey	6	5·89

These figures are well within the latent tetany level.

This first experiment was of necessity of a preliminary nature. It showed the value of milk and whey in supplying a part at least of the calcium in which ordinary white bread is so deficient, but indicated at the same time that the effects brought about by milk and whey were due to something more than the mere addition of calcium. A second experiment was planned to inquire into the nature of this effect.

(B) *Second experiment.* In this experiment an attempt was made to separate the effects due to milk calcium from those due to other milk constituents. Whey bread was not examined this time as there appeared to be no doubt that information gained from milk bread would apply to it equally.

Unsupplemented white bread, bread containing 6 % skim-milk solids and bread to which enough $CaCO_3$ had been added to bring its calcium content to that of the milk loaf were compared. The analyses of the breads are given in Table 12. Calcium carbonate was used this time as this salt was likely to be chosen for universal addition to bread. Groups of three litter-mate male rats weighing 35–51 g. were placed at weaning in individual cages. They were allocated by toss of coin to the three breads. The breads were fed ad libitum as in the first experiment and consumption records were kept. After 3 weeks all animals were given 4 i.u. daily of β carotene. By that time, and for that matter almost right from the start of the experiment, it was clear that the calcium supplement was responsible for only a fraction of the beneficial effect of the milk addition; this is clearly shown in Fig. 2.

From then on several additions were made to the non-milk breads. The first consisted of 3μg. of riboflavin daily, a quantity estimated from the analyses of the breads to represent the amount derived from milk by rats eating approximately 11 g. of milk bread daily.[1] An aqueous solution of riboflavin at *p*H 2 containing 30μg./ml. was prepared freshly every 3 days for this purpose and was checked by daily fluorimetric tests.

The second addition, made after consultation with Dr T. F. Macrae of the Lister Institute, was of a source of the 'filtrate factors' of the vitamin B_2 complex. These factors were almost certainly lacking in white bread; on the other hand skim milk and whey are known to be excellent sources of at least one of them, pantothenic acid (42). They were supplied as a fuller's earth filtrate prepared in the following manner from a by-product of manufacture of the anti-pernicious anaemia principle. 960 ml. of a 1st residue liver extract supplied by Glaxo Laboratories Ltd. (1 ml. $\equiv$ 8 g. fresh liver) was brought to *p*H 3 by the addition of conc. HCl, 128 g. fuller's earth (Fuller's Earth Union specially selected, activated) was added and the mixture allowed to stand for 15 min. with frequent shaking; it was then filtered and washed twice with 50 ml. water. This procedure was repeated twice more and the final filtrate made up to 1280 ml. so that 1 ml. of extract $\equiv$ 6 g. of fresh liver.

The third addition was of 2·5μg. of vitamin B_1. This again was an estimate of the extra amount of aneurin received by the rats getting the milk bread. It will be remembered that our analysis (p. 198) showed that as far as the rat is concerned white bread is relatively more deficient in riboflavin than in aneurin. The next stage in the series of supplements was to equalize the protein content of the breads by the addition of casein (Table 4) to the two non-milk breads. The last change consisted in substituting the 1st residue Glaxo liver extract for the fuller's earth filtrate, thus adding vitamin B_6 to the 'filtrate factors'.

The effects of all these consecutive additions may best be judged from Fig. 2 and Table 16, where the time elapsing between successive additions will also be found.

[1] This quantity may have been too low, cf. p. 198.

To deal first with the unsupplemented white bread, it can be seen that at first it promoted growth nearly as successfully and quite as efficiently as the bread to which calcium carbonate had been added. The riboflavin supplement

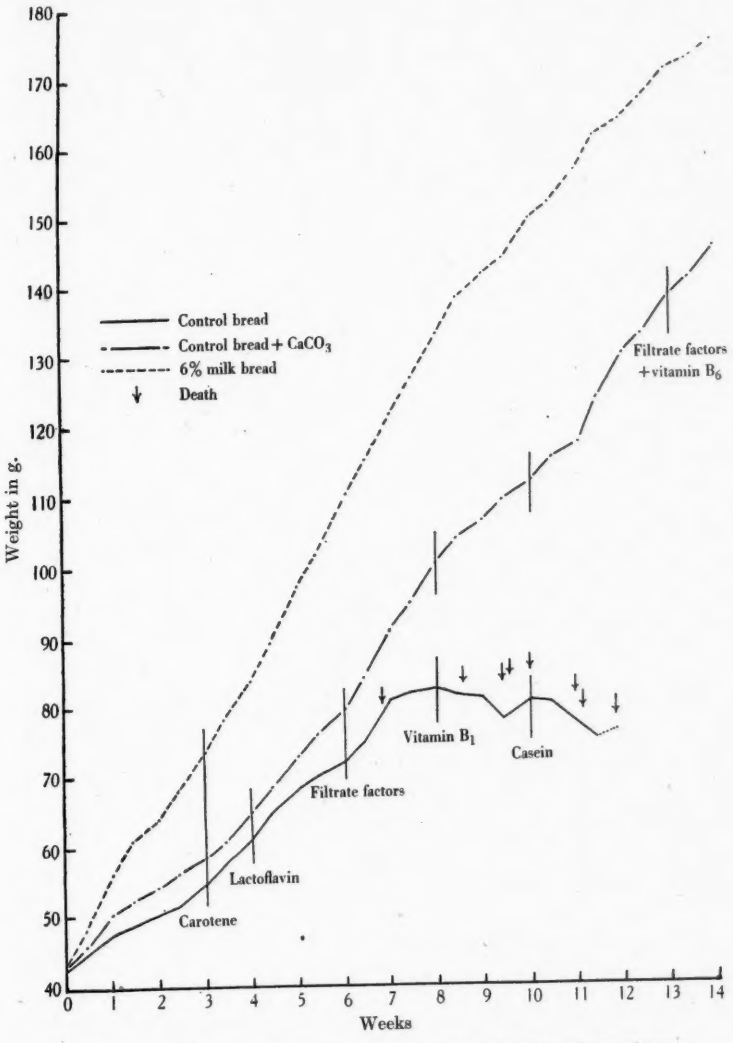

Fig. 2. Growth of rats receiving the various breads. Second experiment.

had no perceptible effect either on the growth rate or on the efficiency of food utilization, and by the time the fuller's earth filtrate was given the effects of the severe calcium deficiency were beginning to tell. From then on the animals remained stationary in weight and all were dead by the 12th week of the

Table 16. *Performance of rats receiving various experimental breads as an exclusive diet (second experiment)*

Rat no.	Initial weight of rat (g.): Start of experiment	1st addn riboflavin	2nd addn 'filtrate factors'	3rd addn vit. B_1	4th addn casein	5th addn 'filtrate factors' + vit. B_6	Final wt. of rat (g.)	1st period, 4 weeks No addition: Bread intake (g.)	g. gain/g. bread intake	2nd period, 2 weeks Riboflavin added: Bread intake (g.)	g. gain/g. bread intake	3rd period, 2 weeks 'Filtrate factors' added: Bread intake (g.)	g. gain/g. bread intake	4th period, 2 weeks Vitamin B_1 added: Bread intake (g.)	g. gain/g. bread intake	5th period, 3 weeks Casein added: Bread intake (g.)	g. gain/g. bread intake	6th period, 1 week 'Filtrate factors' and vitamin B_6 added: Bread intake (g.)	g. gain/g. bread intake	% bone ash (at death
Control bread																				
10188	42	64	74	85	68	Dead	—	233	0·094	134	0·075	130	0·085	—	—	—	—	—	—	28·5
10192	45	63	74	87	82	Dead	—	256	0·070	151	0·073	161	0·081	—	—	—	—	—	—	36·6
10197	40	59	71	75	Dead	—	—	228	0·083	155	0·077	141	0·028	—	—	—	—	—	—	26·8
10217	42	66	78	80	Dead	—	—	282	0·085	168	0·071	124	0·016	—	—	—	—	—	—	30·7
10224	51	61	71	82	80	Dead	—	221	0·045	116	0·086	140	0·079	—	—	—	—	—	—	34·8
10241	44	54	57	Dead	—	—	—	218	0·046	117	0·026	—	—	—	—	—	—	—	—	32·1
10270	44	74	88	95	91	Dead	—	307	0·098	171	0·082	165	0·042	—	—	—	—	—	—	28·8
10278	35	48	64	71	Dead	—	—	215	0·060	142	0·113	136	0·051	—	—	—	—	—	—	29·6
Mean	42·9	61·1	72·1	82·1	—	—	—	274·1	0·073	144·3	0·075	142·4	0·055	—	—	—	—	—	—	30·99
Control bread + $CaCO_3$																				
10189	43	68	75	93	107	126	133	291	0·086	138	0·051	155	0·116	182	0·077	283	0·067	96	0·073	42·2
10193	46	64	76	97	106	132	140	256	0·070	141	0·085	174	0·121	167	0·054	288	0·090	111	0·072	45·3
10194	42	68	81	99	108	135	146	283	0·092	150	0·087	163	0·110	161	0·056	299	0·090	109	0·101	47·8
10220	39	64	83	105	119	147	149	287	0·087	167	0·114	192	0·115	198	0·073	312	0·090	109	0·018	47·8
10225	51	66	79	108	120	141	146	272	0·055	144	0·090	195	0·149	201	0·060	319	0·066	116	0·043	48·1
10239	44	60	74	88	100	128	136	253	0·063	155	0·090	157	0·089	174	0·069	290	0·097	108	0·074	46·6
10269	42	64	85	104	115	147	154	286	0·077	168	0·125	188	0·101	172	0·064	319	0·100	111	0·063	45·6
10276	38	66	83	108	125	149	156	286	0·098	173	0·098	209	0·120	218	0·078	334	0·072	117	0·060	48·8
Mean	43·1	65·0	79·5	100·3	112·5	138·1	145·0	276·8	0·079	154·5	0·093	179·1	0·115	184·1	0·066	305·5	0·084	109·6	0·063	46·53
6 % milk bread																				
10186	42	86	109	133	153	180	183	342	0·129	188	0·122	217	0·111	208	0·096	355	0·076	125	0·024	45·9
10195	46	89	113	134	148	163	166	381	0·113	204	0·118	222	0·095	221	0·063	347	0·043	126	0·024	45·8
10196	43	79	103	121	134	149	156	325	0·111	198	0·121	210	0·086	200	0·065	337	0·045	123	0·057	45·1
10219	40	81	115	136	169	198	206	368	0·111	225	0·151	247	0·085	282	0·117	462	0·063	153	0·052	52·2
10226	51	89	110	133	140	159	161	349	0·109	184	0·114	208	0·111	203	0·034	337	0·056	116	0·017	46·6
10242	43	78	104	124	141	162	162	351	0·100	191	0·136	213	0·094	196	0·087	342	0·061	115	0·000	46·0
10268	44	90	125	153	174	197	200	374	0·123	252	0·139	265	0·106	252	0·083	425	0·054	148	0·020	50·5
10277	38	78	104	130	141	158	165	347	0·115	225	0·116	240	0·108	227	0·048	379	0·045	138	0·051	50·3
Mean	43·4	83·8	110·4	133·0	150·0	170·7	174·9	354·6	0·114	208·4	0·127	227·8	0·099	223·6	0·074	373·0	0·055	130·5	0·031	47·80

experiment. In most cases death was due to respiratory complications probably following collapse or deformation of the thoracic walls.

The rats receiving the calcium carbonate bread did not respond to the addition of riboflavin by faster growth, but the efficiency of food utilization appeared to be improved by the treatment. The administration of the 'filtrate factors' exerted a definite effect on growth rate and utilization of food alike. From this point onwards the rats in this group made at least as economical gains as the rats receiving milk bread. It should be remembered in this comparison, however, that the latter rats were larger and had greater maintenance requirements.

The vitamin B_1 addition was apparently without further effect. It is possible that, as already mentioned, it was not a limiting factor in this case.

The addition of casein to calcium carbonate bread made the rats eating it grow faster. Their rate of gain after this addition was equal to that of the milk bread rats.

The skeletal changes in the rats receiving the 6 % milk bread and the bread containing $CaCO_3$ were much less severe than those observed on the control bread, but rib fractures were present in most animals. It should be remembered that, though for their slow rate of growth the calcium intake of these animals was nearly 'normal'(17), their diet was lacking in vitamin D.

At the end of the experiment all surviving animals were killed by coal gas and the femora and humeri removed for estimation of ash content. All the rats receiving unsupplemented white bread died before the end of the experiment and their bones were removed as death occurred. Table 16 shows the marked difference in ash content between this group and the other two which received extra calcium. On the other hand the difference between the rats given milk or calcium carbonate as a source of calcium was small.

(C) *Third experiment.* The previous two experiments have shown that the nutritional superiority of bread containing milk solids over ordinary white bread is due to several factors, of which calcium is not necessarily the most important. The great value of the riboflavin present in the milk and whey and of the 'filtrate factors' most probably supplied by them were also made evident. In this respect our results with milk bread point in the same direction as Chick's(43) findings in her comparison of wholemeal flour with white flour supplemented with vitamin B_1, minerals and protein.

In the last experiment the 6 % milk bread was taken as a basis of comparison and enough calcium carbonate and casein was added to white bread and to 6 % whey bread to bring their calcium and protein content to that of the 6 % milk loaf.

Eight groups of three litter-mate male rats weighing 35–49 g. were allocated at weaning to the three breads and were placed in individual cages. For the first week all three groups were given ordinary white bread purchased locally. The controlled experiment was then started and the three experimental breads fed. All rats were given 4 i.u. daily of β carotene. In addition the rats receiving

the white bread were given daily 3 μg. of riboflavin, 2·5 μg. of vitamin B_1 and 1 ml. Glaxo 1st residues liver extract equivalent to 6 g. of fresh liver and supplying the 'filtrate factors' and vitamin B_6. All rats were given distilled water to drink. At the end of the experiment they were killed by coal gas and the ash content of their femora and humeri was determined. The results of the experiment are given in Fig. 3 and Table 17. This time the growth of all

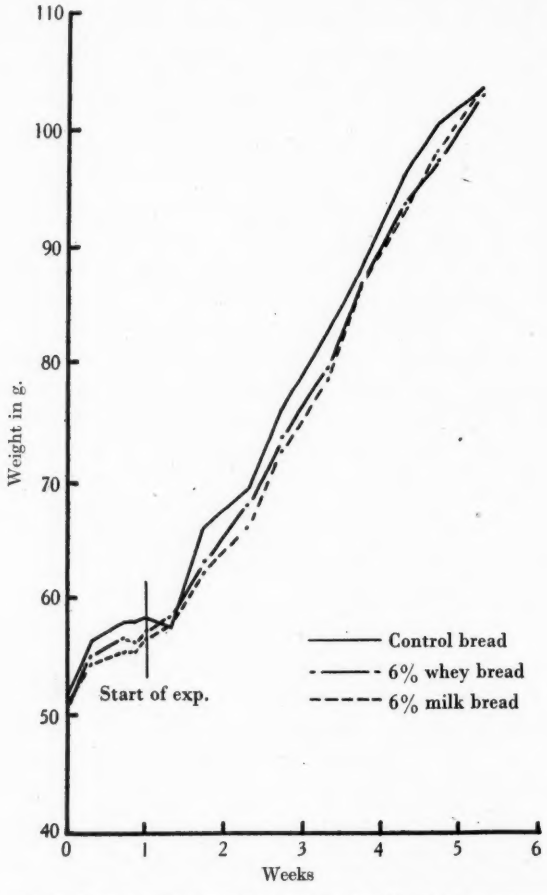

Fig. 3. Growth of rats receiving various breads. Third experiment.

three groups was nearly identical, the efficiency of food utilization almost the same and the bone ash content very similar. This experiment was much shorter than the first two, and therefore no macroscopic skeletal changes were noticed though, as previously, the calcium intake was subnormal (Table 14).

A comparison with the results of the second experiment shows the importance of the vitamin factors added to white bread by the incorporation of milk.

It is also clear that whey is in this respect quite equal to milk and that the differences noted in the first experiment between corresponding milk and whey breads were probably due to differences in calcium and possibly in protein content.

The similarity in bone ash content of the three groups of rats together with the fact that their growth rate was nearly the same indicate that the calcium added as calcium carbonate was as available as the calcium present in milk and whey. This is in agreement with the findings of Tisdall & Drake(44).

Table 17. *Performance of rats receiving various experimental breads as an exclusive diet (third experiment)*

Rat no.	Weight of rat (g.) Initial	Gain	Bread intake (g.)	g. gain/g. bread intake	% bone ash
			Control bread		
11135	55	43	327	0·131	43·2
11142	56	37	293	0·126	45·6
11149	57	58	429	0·135	42·6
11154	56	51	403	0·127	44·1
11163	48	34	280	0·121	40·1
11183	58	45	358	0·126	41·7
11189	64	41	359	0·114	45·9
11195	63	39	346	0·113	44·9
Mean	57·1	43·5	349·4	0·124	43·51
			6 % whey bread		
11133	52	46	340	0·135	41·2
11143	55	32	284	0·113	45·1
11145	59	52	410	0·127	42·6
11155	59	48	353	0·136	44·8
11164	48	36	288	0·125	40·2
11180	62	53	441	0·120	42·3
11188	67	48	416	0·115	45·0
11196	57	48	391	0·123	39·8
Mean	57·4	45·4	365·4	0·124	42·63
			6 % milk bread		
11134	59	56	400	0·140	44·1
11140	52	39	340	0·115	43·1
11144	55	60	402	0·149	42·8
11153	57	51	387	0·132	44·5
11165	47	34	302	0·113	40·2
11182	59	43	364	0·118	41·5
11186	62	44	371	0·119	43·1
11197	61	47	382	0·123	42·5
Mean	56·5	46·8	368·5	0·126	42·73

The experiments just described were carried out between January and August 1940 and they were largely completed before the announcement last July of the proposals of the Ministry of Food to 'fortify' white bread with vitamin B_1 and a calcium salt. We have carried out no direct comparison of the nutritive merits of the 'fortified' loaf with those of milk or whey bread and indeed this would not have been possible as the nature and amount of the calcium addition had not been decided by the time our experiments were completed. There is little doubt, however, that in tests on rats the former would

prove inferior and that the inferiority would be due, as in Chick's(43) experiments with wholemeal bread, to a lack of the B_2 vitamins in white bread.

Even if only 4 % were added to bread more than 160,000 tons annually of dry skim milk would be required to provide for the whole of the bread supply of Great Britain. For this reason it would be absurd to suggest as a war-time measure its compulsory use in all breadmaking.

The marked improvement in the nutritive value of bread resulting from the addition of skim milk and whey is none the less evident from our experiments and we feel that increased use of them in breadmaking in this country should be given serious consideration in more normal times. The extent to which this is being done in the United States is shown by the work of Prouty & Cathcart(45).

For the present we hope that our results will serve to emphasize the great value of dry skim milk and dry whey in war-time dietaries and the need for making as much of them as possible available for general consumption.

Summary

Experiments were carried out to study the effects on the quality and nutritive value of bread of the addition to white flour of roller-dried skim milk and roller-dried whey. Both samples were typical commercial products.

Additions of 2 % of the dried milk could be made without any marked effect on loaf quality or on flavour. The addition of 4 % or more definitely lowered the quality of the bread, the volume being smaller and the crumb more rubbery. As the content of milk was increased above 2 % the flavour of the bread became increasingly distinctive and departed from the normal neutral flavour of water bread.

Up to 5 % of dried whey could be added to the flour without any marked deterioration in the crumb of the bread, although with one flour this quantity decreased the volume by 16 %. At this level, however, the whey imparted a distinct cheesy flavour to the bread.

Attention is drawn to the fact that the effects produced by the addition of dried milk or whey to bread can only be considered in relation to the particular sample used, since other workers have found that modifications in the method of manufacture considerably alter the value of the product as far as its use in bread is concerned. For this reason improved types of dried milk or whey might well lead to their greater use by the baking industry.

The addition of 2 % milk solids doubled, 6 % whey trebled and 6 % milk quadrupled the calcium content of white bread. In hard-water districts a large part of the calcium of white bread is derived from tap water. When white bread was the sole source of calcium for young growing rats, their calcium intake was grossly subnormal. Owing to unavoidable metabolic losses only 60 % of the ingested calcium was retained. The bread calcium was, however, well utilized and is probably as available as that of calcium acid phosphate

and only slightly less so than milk calcium. Addition of dried skim milk or dried whey to the bread increased the percentage retention of calcium. This added calcium was almost completely retained.

A comparison of the biological values and true digestibilities of white bread, 2 % milk bread and 6 % milk bread by the method of Mitchell gave the following respective values: 44·7 and 90·9; 47·6 and 89·6; 49·7 and 88·9. A separate comparison of white bread, 2 % whey bread and 6 % whey bread yielded similarly 47·4 and 92·9; 45·9 and 91·1; 47·4 and 88·9. At most, 15 % of the bread proteins were derived from milk and no supplementary relation was detected at such low levels. Addition of 6 % milk solids increased the 'protein value' of bread by 25 %.

Fluorimetric tests showed that the vitamin B_1 content rose form 1·0 μg./g. dry matter in white bread to 1·3 μg./g. in the 6 % milk or whey loaves.

Riboflavin was similarly increased from 36 to 86–100 μg./g. It is possible that the full extent of the increase was not measured by the fluorimetric test.

Several experiments on rats in which the breads were fed as an exclusive diet showed that the marked beneficial effects of milk or whey additions were due not only to the increase in calcium but also to a large extent to the increase in riboflavin and other members of the vitamin B_2 complex.

We are greatly indebted to Dr T. F. Macrae for the method of preparation of the liver fuller's earth filtrate, to Dr Rowland for the analysis of milk and whey and to Dr Folley for the serum calcium analyses.

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BIENNIAL REVIEWS OF THE PROGRESS OF DAIRY SCIENCE

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SECTION A. PHYSIOLOGY OF DAIRY CATTLE

II. NUTRITION

Feeding standards

Wright & Morris (1) have briefly reviewed the energy requirements of dairy cattle along with those of other classes of stock. They draw attention to the calculations of Brody and of Kleiber, which indicate that the estimation of the maintenance requirement on the basis of the surface area of the body is not well founded. Both these latter authors have abandoned considerations of surface area, and assess the maintenance requirement on the basis of its proportionality to $W^{0.73}$. Brody (2) suggests the formula $Q = 39{\cdot}5W^{0.73}$, where the basal metabolism (Q) is measured in calories and the live weight (W) in pounds. Gaines (3) has suggested an analogous method of calculating the energy requirement for milk production, his formula, derived from an analysis of American data, being $D = 0{\cdot}275F + 0{\cdot}009W$, where D, F and W represent lb. of digestible nutrients, lb. of milk of 4 % fat content, and lb. of live weight respectively. He incidentally concludes in a further paper (4) that the yield of milk, measured in terms of energy, is independent of age and that the so-called 'age correction' is merely an indirect way of allowing for live weight. Wright & Morris (1), while drawing attention to the proposals for a single basis of calculating energy requirements which were made at the Pennsylvanian Conference on Energy Metabolism and which were largely based on Brody and Kleiber's findings, have concluded that the general principles of rationing adopted in Great Britain, as laid down by Wood & Woodman (5), do not warrant revision meantime. They suggest, however, that the maintenance requirement could be safely reduced from 6 to 5½ lb. per 1000 lb. live weight (though in practice this would form an almost negligible reduction in the total ration), but state that the requirement for milk production does not need any modification.

As regards protein requirements, reviews have been published by McCandlish (6) and by Wright (7). Both these authors point out that during the past 25 years there has been a marked general tendency to reduce the earlier standards. From his own experiments McCandlish concluded, however, that there was a real practical difficulty in estimating the protein requirements by ordinary feeding trials, for the protein intake might, he found, be changed considerably without producing any proportionate alteration in the milk yield. This view does not receive support from the experiments of Borland (8), who, in a two-year trial involving twenty-four cows, employed rations of grain at the Haecker standard and at 10 and 20 % below and 10, 20 and 30 % above that standard. Returns in milk production were found to be proportional to the grain fed, even at the highest level. Bartlett *et al.* (9) and Mackintosh (10) in experiments designed to test the effect of lowering the protein standard for milk production from 0·6 to 0·4 lb. protein equivalent per 10 lb. of milk, failed to obtain any reduction in the yield of milk or any loss of condition in the animals, though the rise in milk yield when the animals were put out to grass was greater with those which had been fed at the lower protein level. Cunningham & Addington (11),

by restricting the ration of seven cows to the fodder hegari (a type of sorghum) plus sufficient cotton-seed meal to supply the protein requirement, obtained a 20 % fall in milk yield in the first lactation and a 27 % fall in the second. Braccini (12) compared a ration which gave just sufficient nitrogen to balance the total nitrogen output in the milk, urine and faeces with a more liberal one, and found that an increase in the ration raised the milk yield. Morris & Ray (13) claimed, on the other hand, that a large excess of protein derived mainly from earthnut cake tended to reduce the milk yield and resulted in a lowered biological value of the protein of the total ration. These various contradictions only appear explicable on the assumption that the quality of the individual constituents of a ration are as important as the total quantity of the ration, and that, in addition, it is extremely important in comparing rations to feed at a *minimum* level.

As regards this minimum level, the experiments of Bartlett *et al.* and of Mackintosh confirm the fact that the figure can safely be reduced below 0·6 lb./10 lb. milk. This is also apparent from the results of Morris & Ray, who fed successfully at a 0·4 lb. level, as well as from the collected results reviewed by Wright. There is no doubt that these various findings fully justify the reduction of the official standard to 0·5 lb., the figure which has been adopted in the Ministry of Agriculture's most recent edition of *Bulletin* no. 42 (14).

The feeding of calves

Wise *et al.* (15, 16) have shown that the consumption of excessive amounts of casein by calves produces lethargy, occasional digestive disturbances and an increased plasma nitrogen, but that it does not interfere with growth or appearance. They therefore conclude that the disturbance of mineral metabolism in milk-fed calves is not due to excessive protein ingestion. All calves were given a mineral supplement and cod-liver oil. Leitch (17), in a review, concludes that sprouted fodder and germinated grain have no special value for calf feeding (or for milk production) except in the absence of more usually employed foodstuffs.

McCandlish (18) obtained successful results by feeding calves at 30–40 days of age on reconstituted powdered skim milk with flaked maize as a supplement. A satisfactory growing mixture was whole oats, whole maize, linseed cake, and bran. Archer *et al.* (19) produced normal growth and health in calves after 2–4 days of age, by feeding separated milk or reconstituted skim milk fortified with cod-liver oil. Newman & Savage (20) have found that a level of 20 % dried skim milk in the calf's diet may be reduced to 10 % by the addition of its protein equivalent in the form of yeast or of yeast and soya-bean meal.

Utilization of non-protein nitrogen

With the advent of war in Europe interest has once more been stimulated in the vexed question of the value of non-protein nitrogen compounds for stock feeding. Earlier work was largely concerned with the nutritive value of naturally occurring non-protein nitrogen compounds, frequently termed 'amides'. This term covers, however, not only true amides (e.g. asparagine), but any non-protein nitrogenous substance such as amino-acids and betaines which occur in plant tissues. Estimates of the quantities of such substances vary somewhat according to the method used. Thus Roth (21) compared trichloracetic acid, tannin, uranyl acetate and copper hydroxide as protein-precipitating agents. These reagents gave reasonably concordant results for true protein in various cereal grains but were not so satisfactory when applied to roots and leaves.

'Amides' are of such general occurrence in biological material that in working out a scientific basis of animal rationing some evaluation of them is essential.

Moreover, the large increase in the artificial fixation of atmospheric nitrogen, by making available simple compounds such as urea and ammonium bicarbonate, has re-emphasized the importance of this subject, particularly at a time when protein-rich concentrates are only obtainable with difficulty in the belligerent countries.

The problem of the value of non-protein nitrogen in nutrition, in spite of experiments going back to 1895, has not yet been satisfactorily solved. Krebs (22) has made an exhaustive review of the German literature on the subject up to 1937. He quotes many recent experiments [e.g. (23, 24, 25)] in which subminimal rationing of nitrogen was used and in which milk yields were stimulated by the application of urea. Krebs explains this stimulation of lactation as due to the potential alkalinity of the urea. A basal ration to which urea has to be added in order to bring it up to the protein content necessary for the maintenance of a given milk yield must have a very wide nutritive ratio. Such a ration, according to Krebs, produces a digestive depression as a result of an abnormally high concentration of organic acids in the gut. Hence such a basal ration would normally reduce the milk yield. If, however, the nutritive ratio is restored to normal limits by the addition of urea (but not, be it noted, by the addition of glycine or asparagine) the ammonia generated by the hydrolysis of the urea will remove the digestive depression by neutralizing the excess of organic acids and will therefore allow the milk yield to return to normal. This hypothesis, Krebs admits, involves a second, namely, that the use of urea has disclosed an additional general function of protein, i.e. that of maintaining the correct conditions of acidity in the rumen. In support of this hypothesis, Lenkeit & Becker (26) found that, 30 min. after feeding amide slices to a sheep, there was a rise in the concentration of ammonia in the rumen and that the urea had entirely disappeared.

At the end of his exhaustive review, Krebs summed up by saying that no one had produced evidence of any increase of flesh as a result of urea feeding, and that until such an increase had been demonstrated it could not be said that there was any substitution of protein by urea. However, Hart *et al.* (27) have since subjected urea and ammonium bicarbonate to a very stringent test by comparing them with casein. In a preliminary experiment four male calves were used. The basal ration, made up of yellow corn, starch, timothy hay, steamed bone meal, iodized salt and cod-liver oil, furnished only 6 % protein. This was supplemented for the experimental calves with urea, ammonium bicarbonate or casein in sufficient quantities to raise the crude protein content to 18 %. The control animal showed an increase in weight of 120 lb. in 28 weeks, during which period the ammonium bicarbonate, urea and casein-fed animals showed increases of 170, 220 and 310 lb. respectively. Over a further 14 weeks, during which corn molasses was added to the rations, the respective gains in weight of the four animals were 62, 102, 112 and 128 lb. The animals were slaughtered at 600–700 lb. The moisture, fat and protein contents from the legs, shoulders, ribs and livers were very similar. The experiment was repeated with six heifer calves on somewhat similar rations. One calf received the basal ration alone, one the basal ration plus casein, and a third the basal ration plus ammonium bicarbonate. The remaining three calves received the basal ration supplemented by urea to the extent of 43, 61 and 70 % of their respective nitrogen intakes. Comparable rates of growth were observed in all the animals except the one receiving the basal ration. The weight of this latter animal remained constant for 12 weeks at about 290 lb. The condition of this animal was unsatisfactory, its coat was rough and shaggy, its appetite was poor, and it was listless and constipated. After 12 weeks it was given 1·4 lb. urea per 100 lb. of feed. A remarkable change occurred. The appetite quickened and growth continued for 9 months at the rate of 1 lb. per day, which was but slightly less than for the casein-fed calf (1·2 lb. per day).

Except for a rather large concentration of urea in the case of the animal which ingested 70 % of its nitrogen as urea, blood analyses revealed no significant differ-

ences between the calves. In both the calves which received 61 and 70 % respectively of their nitrogen as urea, there was definite diuresis. The animals showed regularly recurring oestrus. Post-mortem analyses again showed no significant differences. Bacterial counts and the *p*H of the rumen at slaughter were similar. In two calves there was, however, some evidence of damage to the kidneys, and in one the liver showed areas of necrosis. These experiments indicate clearly that both urea and ammonium bicarbonate can be utilized for growth by calves. It may be noted that similar conclusions have been arrived at as the result of experiments with dairy heifers by Bartlett & Cotton (28) who found in a trial involving twenty-one animals and lasting 142 days that urea has a statistically significant effect in increasing the rate of gain of live weight. Fingerling (29), Liliencron (30), Murray & Romyn (31) and Work & Henke (32) have all reported favourably on amides as promoters of growth in ruminants.

Woehlbier & Windheuser (33) found that the nitrogen balances of sheep on minimal rations became negative when beet pulp containing urea was fed. Klein *et al.* (34), however, kept a growing sheep, 50 kg. in weight, in positive nitrogen balance when 6·5 g. of 11·6 g. intake of nitrogen were in the form of ammonium salts.

Turning to milk production, several workers have recently studied the value of non-protein nitrogen compounds. Schmidt *et al.* (35) investigated the availability of urea for the production of milk. In planning their work they assumed a 50 % utilization of urea by the cow. Only one normally fed and five urea-fed animals were used. A 14-day control period, during which all the animals were fed alike, was followed by a 76-day experimental period. During this time the milk yield of the normally fed animal fell from 25·9 to 18·1, i.e. by 7·8 kg., and that of the experimental animals from 26·7 to 17·6, i.e. by 9·1 kg. The average yields in the course of the experimental period were 22·5 and 22·1 kg. per animal per day respectively. The experimental group lost 4 kg. in body weight per animal in the experimental period, while the normally fed animal gained 5 kg. At the end of the experiment a deficient group of animals, the milk yields of which decreased from 18·4 to 14·4 kg. in 10 days, was also included in order to show that the control animal had not been overfed.

Experiments by Ehrenberg *et al.* (36) with four groups of eight cows were planned in a similar fashion to those of Schmidt *et al.*, and confirmed previous German work as to the protein-sparing effect of urea. Ehrenberg and his colleagues claim that glycine also has a sparing effect. Ehrenberg *et al.* (37), as a result of another experiment of the same type which involved some twenty cows, came to the conclusion that urea was not utilized to the extent of 50 % when it replaced the protein required for 7–8 kg. milk out of a total of 17 kg., but that when it only replaced the protein requirement for 4 or 5 kg., its utilization by the animal was greater than 50 %. Ehrenberg & Steinhardt (38) found ammonium bicarbonate inferior to oil-cake as a milk-producer.

Nehring (39), in further experiments, came to the same conclusion as Ehrenberg *et al.*, i.e. that there is a limit to the extent to which the protein of the production ration can be replaced by urea. Nehring found a beneficial effect when urea was given in sufficient quantity to replace the protein of the production ration for 5 kg. milk, but a deleterious effect when urea was given for 10 kg. milk. In these experiments, in which eighteen cows were used, the protein equivalent of the urea was calculated on the basis of a 50 % utilization.

Results of experiments of Richter & Herbst (40) support the claim of Ehrenberg *et al.* that glycine has a protein-sparing effect. Richter & Herbst used four groups, each of eight cows. One group was deprived of part of the protein of the production ration, a second was normally fed, a third had 50 % of its production ration replaced by urea, and in the fourth group 50 % of the production ration was replaced by glycine. The use of the supplements resulted in a fall in milk yield which was less

evident with glycine than with urea. All four groups of animals lost weight in the course of the experiment, but the glycine-fed animals maintained their weight best, followed by the normally fed control animals. The urea animals and the animals deprived of part of their production ration lost most weight. The superiority of glycine over urea in these experiments argues against Krebs's hypothesis that urea acts by inhibiting digestive depression.

Schmidt & Kliesch (41) have tested the effect of urea, using identical twin heifers. They found that replacement of half the production ration by urea caused only a slight reduction in the yield of milk and butterfat of the experimental animal as compared with its twin sister. Repetition with a second pair of identical twins (42) in an experiment lasting for 4 months confirmed the results obtained with the first pair. These workers conclude from their results either that present feeding standards are too high or that urea can replace some of the protein necessary for production.

Mineral metabolism

(*a*) *Calcium, phosphorus, vitamin D and ultra-violet light*

An informative review on the metabolism of calcium and phosphorus with special reference to bovines has been prepared by Otto (43).

Rickets can be produced experimentally (Shohl (44), Dunlop (45), Bachman *et al.* (46) and Jones (47)) by diets low in calcium or phosphorus or both, or by diets which lack vitamin D and which have at the same time a ratio of calcium to phosphorus greatly different from that found in bone. Animals receiving adequate vitamin D (natural or artificial) or undergoing adequate ultra-violet irradiation (natural or artificial) can withstand calcium and phosphorus deficiencies or an unfavourable Ca/P ratio more effectively than those with an inadequate supply. Citric acid can also aid in the retention of calcium and phosphorus (Hathaway & Meyer (48)).

A normal animal can resist the adverse effects of calcium or phosphorus deficiency for a considerable time without showing any symptoms of either rickets or tetany. This is because, if the animal does not receive enough calcium or phosphorus in its food, it can utilize some of the vast skeletal stores of calcium phosphate. On a restricted mineral intake rats (Gaunt *et al.* (49), Bachman *et al.* (46)) can grow bones of a mineral content which reflects the deficiency of the diet in calcium and phosphorus. The same is true of pigs (45) and of cattle (Theiler & Green (50), and Mead & Regan (51)). Not only will deficient diets prevent growing animals from producing a fully mineralized skeleton, but any deficiency in adult life may remove calcium and phosphorus from the skeletal stores. Such a state of affairs seems to be the normal accompaniment of lactation in both the cow and the human (Garry & Stiven (52)). Increased retentions which occur later serve to replenish these skeletal stores. Moreover, Rottensten (53) has shown that rats on a diet unusually low in calcium and phosphorus retain those elements with greater efficiency than normally fed controls when both they and their controls are fed a normal diet. Hence the skeleton can act as a kind of buffer system with regard to supplying the calcium and phosphorus to the soft tissues, depleting itself when the need for these elements is urgent (i.e. during lactation or when the diet is poor in them), and replenishing itself when the needs are easily covered by the diet. Even the relatively aged skeleton can store calcium and phosphorus (Adams *et al.* (54)) when abundantly supplied with these elements. It is not always realized, however, that the skeleton cannot liberate one of the elements calcium and phosphorus without liberating an equivalent amount of the other. Thus the bone salts, even in a very fragile skeleton, have the same composition as those of the skeleton of a normal animal. It is for this reason that the roles of calcium and of phosphorus in nutrition cannot be considered separately. Neither can they be considered in ignorance of the animal's previous nutritional history (53, 55).

Theiler's work showed that in cattle fragile bones may result from aphosphorosis. Becker *et al.* (56) have shown that in cattle calcium deficiency may lead to the same result. Cows with large milk yields were shown by Becker *et al.* to suffer from spontaneous fractures of bones when kept on calcium-deficient diets. Nitsche (57) and Weakley & Dustman (58) have presented data on the variation of calcification of the skeleton of cows and of wild deer.

In growing steers which were fed rations containing calcium and phosphorus in the rate of 2:1, Otto (43) found that 6 g. of phosphorus, an amount less than the full requirement, led to a very efficient retention of calcium and phosphorus. At this level of phosphorus intake there was no significant difference between the retentions of the phosphorus from dicalcium phosphate or from bone-meal. The phosphorus of disodium phosphate was, however, somewhat more efficiently retained. Long-term experiments, at the end of which the animals were slaughtered, showed that rations containing 8·5 g. phosphorus and 16·4 g. calcium were adequate, while rations containing 5·8 g. phosphorus and 12 g. calcium were not. The adequacy of a ration was judged by its effect on the inorganic phosphorus of the blood, on mineral balances, and on the composition and structure of the bones. A close correlation was found between the breaking strength of the fresh bones and their mineral content. In heifers 10·5 g. phosphorus and 23 g. calcium permitted retentions of 6·6 g. phosphorus and 9·7 g. calcium. That these intakes were adequate for heifers was demonstrated by balances and by blood and bone analyses.

Beeson *et al.* (59) produced symptoms of aphosphorosis in fattening cattle by means of a ration consisting of blood meal, beet, limited alfalfa and corn silage. The ration contained 0·12 % phosphorus. The addition of 0·66 % of bone-meal to the ration corrected the deficiency, but cotton-seed meal containing an equal amount of phosphorus was less effective. From these experiments the phosphorus requirement of fattening beef calves works out at 8–13 g., and that of beef steers at 2 g./100 lb. live weight per day.

Archibald & Parsons (60) and Hepke (61) have attempted to alter the composition of the milk by dietary means. Archibald & Parsons, who added disodium phosphate to the rations of eight cows, found no change in the yield or composition of the milk. Hepke (61), who fed goats with monocalcium phosphate, found an increase of inorganic phosphorus, a slight rise of chlorine but no effect on the yield or the calcium or the organic constituents of the milk. Van Landingham *et al.* (62) have reported on the inorganic phosphorus of the blood of twenty-two Holstein dairy cows. Samples were taken at intervals of 8 weeks over a period of 2 years. There was a lowering of blood inorganic phosphorus during the last 3 months of the second pregnancy and during the first 3 months of the second lactation. A similar phenomenon was observed in the third cycle of pregnancy and lactation. Kuhner (63) fed 600 g. of ground oats and 1·5 kg. of meadow hay to each of four goats. Supplements of calcium chloride, calcium carbonate or calcium phosphate failed to influence either the amount or the composition of the milk.

The Vermont Agricultural Experiment Station (64) reports a long-term investigation of the calcium and phosphorus losses of two cows during lactation, but the significance of these losses cannot be judged because the authors, though they report the total content of the calcium and phosphorus in the carcass at slaughter, do not give either the percentage of these elements in the bones or any data from which this percentage can be calculated.

Becka & Krizenecky (65) tested the effect on milk secretion of certain mineral supplements containing calcium. A supplement containing calcium and magnesium phosphates and carbonates appeared to increase both the output and the percentage of fat but had no effect on the total yield. A second supplement, a mixture of calcium and magnesium salts and lactic and fatty acids, was without any effect. Marek

et al. (66) have reported a case of osteoporosis in a pregnant cow due to a ration poor in phosphorus and rich in calcium. Morris & Ray (67), in experiments with sheep, goats and cows, showed that fasting is not accompanied by any extensive katabolism of bone.

Riddell *et al.* (68) analysed the blood of dairy cattle in America to ascertain the effect of an abnormal succession of dry seasons. Owing to lack of pasture, much low-grade roughage (principally sorghum) had been fed to the animals on the thirty-six farms from which these authors collected blood samples. The following average figures were obtained: calcium, 10·55 mg. % (range 8·8–13·2); phosphorus, 5·35 mg.% (2·0–10·3); carotene, 0·394 mg. % (0·018–1·57). Except where symptoms of abnormal appetite had been reported the condition of the animals gave little indication of the mineral level in their blood. The condition was, however, reflected in the blood carotene.

Converse *et al.* (69) compared the gains in body weight of nine heifers receiving timothy hay in their rations with seven comparable heifers receiving bone-meal in addition. The experiment extended from the sixth to the eighteenth month of the animals' lives. During that time there was no significant difference between the gains in weight of the two groups. In a further experiment two calves were fed grain and timothy hay and another two calves grain and alfalfa hay. The calves were killed at 12 months of age. The percentages of ash in the dry fat-free humeri of the timothy calves were 60·1 and 59·5, while the percentages for the alfalfa calves were 60·0 and 60·1.

The significance of the phytin normally present in cereals still remains a mystery. Palmer & Mottram (70) attribute the rachitogenic effect of cereals to their disproportionate Ca/P ratio rather than to a diminution of the availability of phytin-phosphorus. Day (71) finds that the beneficial effect of citrates does not assert itself in the absence of phosphorus-containing complexes such as phytin in the diet. Krieger *et al.* (72) find that in a cereal-free ration inorganic phosphorus is more readily assimilated than phytin-phosphorus, though vitamin D enhances the assimilation of both forms of phosphorus. In a later paper (73) the same authors found that calcium phytate and calcium carbonate were equally available, and that vitamin D improved the availability of each to the same extent.

The physiological action of vitamin D is still a matter of conjecture, it being as yet impossible to decide whether it exerts its major effects by increasing intestinal absorption by enhancing the rate of calcification of bone, or by a combination of both these processes. Thus Shepherd & Macy (74) observed a decreased retention of nitrogen, sodium and phosphorus accompanied by an increased retention of calcium when vitamin D intake was increased through irradiation of dietary milk powder. This observation indicates a favouring of the metabolism of the bone as against that of the soft tissues, for some 98 % of the body's calcium is in the skeleton. Cohn & Greenberg's (75) results with radioactive phosphorus also showed an increased uptake of mineral by the bones in the presence of vitamin D. However, Smith & Spector (76, 77) have shown that feeding of mineral oil inhibits the antirachitic action of alimentary vitamin D and of ultra-violet light as well. It is hard to explain these observations without supposing that vitamin D, whether ingested or produced in the animal by irradiation, has the effect of improving intestinal absorption.

Olson (78) found that absence of sunlight failed to cause any significant decrease in the milk yield of cows. Kuhlmann & Gallup (79) produced rickets in calves by feeding them a ration of cotton-seed meal, beet-pulp, bone-meal and vitamin A. Either replacement of the beet-pulp by prairie hay or allowing the animals access to sunshine were adequate preventives. Wallis (80) compared rations containing alfalfa hay, or beet-pulp or prairie hay as the source of vitamin D. Alfalfa, due to its larger D content, was much more effective than prairie hay in increasing the D potency

of butter produced by the beet-pulp ration. The same author (81) found alfalfa leaves to be much more potent than stems.

(*b*) *Other minerals*

Reviews of the metabolism and nutritional significance of magnesium by Duckworth (82) of trace elements by Godden (83), Underwood (84) and Kehoe *et al.* (85) have been published. Fluorosis has been reviewed by Greenwood (86) and by Peirce (87). The role of metallic elements in blood formation has been reviewed by Schultze (88).

Many papers have appeared recently concerning deficiencies of copper and cobalt in ruminants. According to Hopkirk & Grimmett (89) the sheep's requirement of cobalt per week is 1 mg., though even this small amount is not supplied by some pastures. Sjollema (90) has reported a disease in cows and goats living on freely drained sandy soil which, from the low content of copper both in various organs of the animal and in the local pastures, he concluded to be a copper deficiency. Thijn (91) has reported the occurrence in cattle of cases of nutritional hypochromic anaemia curable by large doses of iron and copper. Askew (92*a*) has found cobalt deficiency in sheep at Sherry River, Nelson, New Zealand. Breeding ewes were kept healthy by 9 mg. cobalt per week. When pastures contain 0·01–0·04 p.p.m. cobalt, sheep are adversely affected. According to Hopkirk & Grimmett (89) the safe limit for cattle is somewhat higher, i.e. 0·04–0·07 p.p.m. Patterson (92*b*) reports that pastures near Dartmoor lack cobalt.

In 1938 Keilin & Mann (93) isolated haemocuprein from the erythrocytes of the ox, and at the same time obtained evidence of a copper-containing protein in the plasma. The physiological functions of these proteins remain obscure. Recently the same two authors (94) have isolated a highly concentrated preparation of carbonic anhydrase, also from ox blood. They find that carbonic anhydrase is a protein containing zinc as an essential part of its molecule. This is probably the most important physiological discovery regarding the function of zinc in the animal organism which has been made since the demonstration of its presence in crystalline insulin.

Matthews *et al.* (95) have demonstrated with a large herd of cows that potassium iodide added to the feed causes a considerable increase (7–26 times) in the iodine in the milk. These authors did not, however, investigate the partition of the iodine in the milk to find out whether any had passed into the milk protein (96, 97).

Archibald *et al.* (98) have described a disease of cattle in Massachusetts which resembles the bush-sickness of New Zealand and which can be cured by iron ammonium citrate drenches. The authors ascribe the cause of the disease to lack of iron in the indigenous forage, but take no account of the more recent work (already quoted) in Australia and New Zealand on the importance of cobalt.

It is now known that the animal can cover its requirement for sulphur from protein (the ruminant can apparently synthesize thiamin (99)). It is often the practice in nitrogen balance experiments to estimate sulphur balances as an additional index of protein metabolism. While theoretically sound, this procedure is attended with great technical difficulty, since it still does not seem to be generally realized that most of the published methods for estimating total sulphur are, in spite of perfect agreement of replicate analyses, quite inaccurate unless used under conditions not usually applicable in routine analysis. Thus the present reviewer has found many of the methods published in the literature to be unreliable even for the purpose for which they were designed, e.g. those described in papers (100) and (101). The chief difficulty is to achieve complete oxidation of the material so that all the sulphur is transformed into sulphate. Critical technical comparison of methods of determining sulphur in biological material have been made by Barlow (102), Marston (103) and Masters (104). Barlow recommends combustion in a stream of oxygen. Marston

recommends combustion in compressed oxygen in the calorimetric bomb. Masters (104) has adapted the method of ter Meulen (which involves reduction of the sulphur to hydrogen sulphide) to the analysis of foods. Masters & McCance (105) have published a list of the sulphur contents of foodstuffs analysed by Masters's method. Marston gives many analyses of fodder, faeces and urine.

In spite of the essential nature of the boron in plant nutrition there is no evidence of its being required by animals. Hove *et al.* (106) found that no benefit resulted from adding boron to mineralized milk for young rats.

(*c*) *Toxic minerals*

Mineral toxicoses naturally occurring in stock are those due to fluorine, molybdenum and selenium. Evans *et al.* (107) have examined the fluorine content of milk and casein. By analysis of bones they have shown that calves obtain most of their fluorine after weaning but that they also receive a little in their dam's milk. Fluorine in small amounts is also able to cross the placenta (107).

Molybdenum is peculiar in being beneficial to some plants, algae and moulds, but toxic even in traces to animals. Thus molybdenum is required by the ubiquitous fixer of atmospheric nitrogen, *Azotobacter* (108). It is apparently essential for the normal growth of tomato seedlings (109). It is also required by the mould *Aspergillus niger* (110). The presence of molybdenum in pastures in Somerset has, however, been shown by Ferguson *et al.* (111) to be the cause of their 'teartness', i.e. the capacity to produce a violent form of scouring.

A series of papers on the toxicity of selenium to stock has appeared in America since it was discovered that 'alkali disease' of cattle (112) is in reality selenium poisoning due to ingestion of selenized plants. Here again certain plants commonly found in selenized areas appear to benefit from the presence of this element (113), (114), and Perkins & King (115) report a stimulation of the growth, harvest weight and early germination of wheat by 2·5 p.p.m. of selenium. It has been found that arsenic combats the toxicity of selenium (116) as also does increasing the level of protein in the diet (117). Gortner (118) has found that lactalbumin can protect an animal against selenium poisoning, though gelatine and edestin are without effect. It has been shown that the selenium in toxic plants is a constituent of the protein, and it seems probable that it is present as selenocystine, i.e. a compound in which the sulphur is replaced by selenium. It has long been known that phenyl halides are detoxicated by the body as cysteine compounds (i.e. as mercapturic acids) and it is therefore interesting to find that Moxon *et al.* (119) have increased the rate of excretion of selenium from dogs and cattle by administering bromobenzene orally. Evidently a selenomercapturic acid is excreted.

Vitamins

General articles concerning the vitamin requirements of farm animals (including cattle) have been published by Wright & Smith (120) and by Guilbert *et al.* (121). Hart has written a review on vitamin A deficiency and the requirements of farm mammals (122). He gives the minimal requirement of cattle as 5·1–6·4 γ (21–27 I.U.) of vitamin A per kg. body weight per day or 26–33 γ (43–55 I.U.) of carotene per kg. body weight per day. Ward *et al.* (123) gave 20–30 γ carotene per lb. weight per day as the requirement of calves. Boas-Fixsen & Roscoe (124) have published a rather extensive table of the content of vitamin A, thiamin, C, D and riboflavin in various foodstuffs, while Bacharach (125) has published a table of nicotinic acid contents of foods. In these tables the reference to the original literature is given for each figure quoted.

(*a*) *The fat-soluble vitamins*

Hart gives a detailed account of the symptoms which develop in cattle as a result of vitamin A deficiency. These are night-blindness, inco-ordinated movement, paresis, loss of weight and appetite, diarrhoea and corneal keratitis. The symptoms take a long time (over 200 days) to appear and are even then not constant in their appearance. Post-mortem examination shows, in very deficient animals, a snow-white body-fat and only traces of vitamin A in the liver. Hart does not mention how, in testing cattle for night-blindness, allowance is made for unsuspected minor inco-ordination of movement. This is of some importance, for Irving & Richards (126) have found by histological examination that in rats neural lesions, particularly of the medulla, are the first signs of vitamin A deficiency, while Thomson *et al.* (127) have shown that in humans the correlation between vitamin A deficiency and night-blindness is by no means perfect. Experiments of various workers (122, 128) show that recovery from the visual effects of vitamin A deficiency is a slow process.

Congenital blindness occurs in the young of mothers on a diet deficient in vitamin A (129). Vitamin A deficiency has specific effects on cranial nerves. Thus in young animals blindness often occurs, facial paralysis is recorded by Hart (122), and Mellanby (130) has produced deafness in puppies by withholding vitamin A. Mellanby ascribes these lesions of cranial nerves to hypertrophy of the bone surrounding them. Moore (131) asserts that they may be caused by the increase of intracranial pressure which results from vitamin A deficiency. Wolbach & Bessey (132) state that lesions of the cranial nerves are due to continuation of growth of the nervous system accompanied by a cessation of the growth of bone. Moore & Sykes (133, 134) have investigated the rise of intracranial pressure which accompanies vitamin A deficiency in calves. They find that the increased pressure is about three times the normal, and that it is accompanied by lowered values for carotene in plasma, by night-blindness and by choking of the optic disk. On return to normal diet the cerebro-spinal pressure slowly returns to normal (134).

Sutton *et al.* (135) in investigating vitamin A deficiency in male calves have found, in addition to symptoms noted by earlier workers, a degeneration of the germinal epithelium, an absence of spermatozoa in the epididymis, and histological changes in the hypophysis. In a trial involving eighty-six calves an increased incidence of pneumonia was observed in the deficient animals. Converse & Meigs (136) state that during the latter part of gestation the cow needs 80–100 mg. of carotene per day to prevent still-birth.

Phillips *et al.* (137) kept two calves on rations which were identical except that 45 γ per lb. of body weight of crystalline carotene was fed to one of them. In 29 weeks the carotene-fed animal doubled its weight; the deficient animal increased in weight from 200 to 285 lb. in the first 12 weeks, but thereafter declined to its initial weight of 200 lb. Towards the end of the experiment the deficient animal became night-blind, but optic stenosis was not observed. By comparison with the control, the deficient animal exhibited a reduction of the riboflavin content of the liver and a marked reduction of both plasma and urinary ascorbic acid. The clotting time of the blood of both animals was longer than normal, but on each of two occasions 28 days apart the administration of vitamin A caused it to be markedly reduced. The authors conclude from this latter observation that cattle probably require vitamin K. At autopsy no lesions were found in the control animal, but hepatic, pulmonary, renal and neural lesions were found in the deficient animal.

Avitaminosis A was produced in calves by Flora *et al.* (138) by feeding rations containing low-grade timothy hay or cotton-seed meal. Fresh carrots, dried alfalfa or a carotene concentrate were instrumental in overcoming the deficiency. Lowered blood carotene due to the deficiency responded quickly to medication. Additional

references to the effect of such a deficiency on the carotene content of blood will be found in (139), (140) and (141) and of liver in (142). There is also a reference to the reduction of growth rate in heifers (143).

(*b*) *The water-soluble vitamins*

More evidence has been obtained as to the power of the ruminant to synthesize the various members of the vitamin B complex, though naturally it is difficult to decide which of these members the ruminant really needs. McElroy & Goss (99, 144, 145) have demonstrated the synthesis of members of the vitamin B complex by sheep after 30 days of continuous feeding of a deficient ration. The ration contained per g. $<0{\cdot}4\,\gamma$ B_1, $<0{\cdot}3\,\gamma$ riboflavin, $<0{\cdot}2$ chick units pantothenic acid, $<1{\cdot}5\,\gamma$ B_6. After 30 days the rumen contained per g. $5\,\gamma$, $20\,\gamma$, 5 chick units, and 6–$10\,\gamma$ of these factors respectively. These authors fed the same diet to a cow with a rumen fistula. After $4\frac{1}{2}$ months the cow gave birth to a normal calf. For 43 days the calf was fed solely on the dam's milk and remained in excellent health, gaining 1·7 lb. in weight per day. The contents of the cow's rumen contained $70\,\gamma$ pantothenic acid per g. of dry matter. The dry matter of the cow's milk contained $18\,\gamma$ per g. There was $1{\cdot}4 \pm 0{\cdot}15\,\gamma$ riboflavin per g. of dry matter of the milk, while milk from cows on a normal ration averaged $1{\cdot}5 \pm 0{\cdot}25\,\gamma$. Tested for nicotinic acid the rumen contents gave $150\,\gamma$ per g. of dry matter, while in the original ration there was none determinable. McElroy & Goss (146) have also shown, by comparing the cow's intake of riboflavin with the output in the milk, that the cow synthesizes this vitamin. In the same paper they show that vitamin K is synthesized in the rumen of the cow. In a later paper (147) they show that in one cow the output of pantothenic acid was twice as great as the intake.

Hunt *et al.* (148) have demonstrated the synthesis of riboflavin in the rumen, but their evidence for that of thiamin is not conclusive.

Pearson *et al.* (149) fed to lambs for 8 months a diet which produces black tongue in dogs. They demonstrated the synthesis of nicotinic acid by measuring its continuous excretion in the urine.

With regard to the synthesis of thiamin (B_1, aneurin) it should be pointed out that assay for the separate thiazole and pyrimidine constituents of the thiamin molecule would be of more value than assay for thiamin itself because pigeons (150) can complete the synthesis of thiamin from these constituents.

Knight *et al.* (151) claim to have demonstrated the destruction of ascorbic acid in the rumen of a cow with a fistula. Their experiment does not, however, preclude the possibility of loss of the acid by passing on to the abomasum. They also state that ascorbic acid incubated in vitro with rumen contents is destroyed rapidly. Dairy cattle can synthesize ascorbic acid (152).

E. C. Owen

The Hannah Dairy Research Institute,
Kirkhill, Ayr.

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SECTION F. MILK-BORNE DISEASES

Introductory

Outbreaks of disease transmitted through the agency of milk continue to occur. In this country, the value of milk as a foodstuff for children and nursing mothers has been particularly emphasized. Furthermore, as a result of war conditions, certain sections of the population have been transferred from large towns, where adequate facilities for pasteurization were available, to small towns and country areas where raw milk only can be obtained. The possible dangers to health resulting from such changes have been noted by many medical authorities, and, in particular, by the Royal College of Physicians (1), which passed a resolution recommending that the attention of the Ministry of Health should be drawn to the fact that large numbers of children, who have been brought up on pasteurized milk in the towns, are now receiving raw milk in reception areas to which they have been evacuated, and that the Ministry should be asked to do all in its power to secure the co-operation of the Milk Marketing Board, and of the medical officers of health, to arrange for supplies of pasteurized milk to be made available in all areas where there are sufficient children to demand it. Further, in the opinion of the College, members of the medical profession might usefully advise parents and guardians of children of the risk of infection which attends the use of raw milk, and of the value of pasteurization, or, alternatively, of boiling the milk in removing these risks. It should be stated, however, that up to the present time no large outbreaks of disease have been reported in the medical press. Infections due to tubercle bacilli would, however, remain latent for some time, and several years must elapse before a possible increase in tuberculosis, due to the bovine type, is evident.

There can be no doubt that efficient pasteurization is an effective safeguard against milk-borne infections. Medical officers of health are practically unanimous in their attitude towards this aspect of the problem of safe milk. Savage (2) has no bias in favour of pasteurization if a safe milk supply can be produced in other ways, but, on the other hand, he has emphasized the fact that efficient pasteurization is an effective barrier to the transmission of disease by milk. Macgregor (3) has strongly advocated the pasteurization of all milk, including T.T., since the risk of transmitting disease is too great to leave even the best-quality milk unpasteurized.

The nutrition subcommittee of a division of the British Association for the Advancement of Science (4) discussed problems connected with the present milk policy, and suggested that compulsory pasteurization might actually so increase public confidence that the amount of milk consumed per head of population would be increased. A resolution was passed advocating that milk supplies to towns with 20,000 inhabitants or more should be subject to compulsory pasteurization, and, in view of the withdrawal of the Milk Industry Bill, it was also suggested that action might be taken against retailers at common law, where it could be proved that the milk supply caused disease. If this proved effective in a few instances, it might do much to impress on the retailers the necessity for effective pasteurization as a means of self-protection.

It would not be impossible, even at the present time, to make arrangements for the distribution of milk pasteurized by the holding process in some areas, and to arrange for short-time high-temperature pasteurization to be carried out in other centres. Not only so, but the phosphatase test of Kay and Graham has been proved to be of immense value in the control of pasteurization. Kay (5) has recently stated that the phosphatase test is equally effective with both holder-pasteurized and H.T.S.T. pasteurized milk. The test is now so precise that it will indicate, with

certainty, in the holder process, a drop of 1·5° F. in the holding temperature, or a diminution of 10 min. only in holding time, or a leakage of as little as 0·2 % of raw milk into the pasteurized bulk.

The foregoing observations apply practically entirely to Great Britain, but in the United States and Canada public health authorities are equally convinced of the necessity for producing clean milk in the first instance, and safeguarding the milk, so produced, by pasteurization.

Epidemiology

Surveys of milk-borne disease have recently been made in two countries—in the U.S.A. and in Canada—and from the former the report of the Committee on Communicable Diseases affecting man has been published by Merchant (6). This paper

Table 1. *Milk-borne epidemics in United States*, 1923–37

Year	Typhoid fever	Sore throat	Scarlet fever	Gastro-enteritis	Other epidemics	Total epidemics	Total cases	Total deaths
1923	15	1	6	0	1	23	834	36
1924	35	1	5	2	1	44	1,552	67
1925	33	6	4	0	1	44	1,739	56
1926	51	6	5	4	2	68	3,364	94
1927	26	0	4	1	5	36	954	91
1928	26	3	8	3	7	47	2,196	120
1929	31	8	10	1	1	51	2,332	53
1930	30	9	2	5	2	48	1,968	56
1931	22	8	1	2	1	34	1,398	24
1932	23	3	6	1	0	33	642	28
1933	25	7	4	6	6	48	1,426	40
1934	26	8	3	7	1	45	1,787	42
1935	16	9	2	16	0	43	1,829	21
1936	15	6	16	5	4	46	1,565	28
1937	15	3	11	14	0	43	1,705	11
Total	389	78	87	67	32	653	25,291	767

Table 2. *Outbreaks of milk-borne disease in Canada*, 1912–37

Disease	No. of outbreaks	No. of cases	No. of deaths
Typhoid fever	47	6701	692
Paratyphoid fever	6	557	7
Scarlet fever	7	192	0
Septic sore throat	3	584	4
Undulant fever	0	965	0
Total	63	8999	703

gives a comprehensive summary and analysis of milk-borne outbreaks of disease from 1923 to 1937 inclusive. Table 1 summarizes the main findings. Typhoid fever holds the first place, since more than half the outbreaks attributable to milk are due to this cause; streptococcal infections, streptococcal sore throat and scarlet fever are the next most prevalent, and gastro-enteritis, which presumably includes both *Salmonella* and staphylococcal food poisoning, is third on the list. From Canada, the paper by Defries (7) emphasizes the fact that the number of sporadic cases of milk-borne diseases undoubtedly greatly exceeds the number of cases reported in outbreaks, and that many small outbreaks are not reported. Table 2 summarizes the occurrence of the various infections in Canada between the years 1912 and 1937. The list is not so comprehensive as that given by the U.S.A. Committee, but here again typhoid fever has been the most serious milk-borne disease, and is associated with the greatest mortality rate. In a third paper, Frank (8) discusses disease outbreaks resulting from faulty environmental sanitation in the U.S.A. during the year

1938, the survey being initiated by the Public Health Service. This survey has provided some very interesting material, and, as is seen in Table 3, foods other than milk were a more prolific source than were milk or water supplies, although water supplies produced the largest number of actual cases. This result was largely fortuitous, however, since no less than 29,250 cases of gastro-enteritis occurred in one city, due to an infected water supply. Table 4 shows the type of milk, or milk product, identified as the source of infection, and here again no less than thirty-eight out of forty-two outbreaks were due to raw milk, and only one to milk which was said to have been pasteurized. Further, the paper again shows that outbreaks of typhoid fever occurred more often, and caused more deaths, than any other infection, although, on the other hand, the actual number of cases of streptococcal infections and gastro-enteritis greatly exceeded the total number of cases of typhoid fever. Furthermore, only 12 % of the outbreaks occurred in communities of more than 10,000 of a population, and this would seem to indicate that the milk supplies of

Table 3. *Total outbreaks, cases and deaths*, 1938, *by vehicles*

Vehicle	No. of outbreaks	No. of cases	No. of deaths
Water supplies	48	31,693	17
Milk and milk products	42	1,685	27
Other foods	70	2,247	25
Unidentified vehicles	8	882	3
Total	168	36,507	72

Table 4. *Outbreaks transmitted through milk and milk products*, 1938, *by kind of supply*

Kind of supply	No. of outbreaks	No. of cases	No. of deaths
Sweet milk: raw	37	1462	27
pasteurized	1	100	0
Buttermilk: raw	1	10	0
pasteurized	0	0	0
Ice cream	2	96	0
Cheese	1	17	0
Total	42	1685	27

small communities are not so safe as those of large communities, the explanation being probably that large towns have pasteurizing plants capable of dealing with a greater percentage of the raw milk supplies.

Streptococcal infections

There have been few recorded outbreaks of streptococcal infections, either streptococcal sore throat or scarlet fever. In those which have occurred, the features of the epidemics have been, first, a preponderance of victims over the age of 15 years, and, secondly, the age distribution of the cases has closely followed the age distribution of the population generally. In contrast, in outbreaks of scarlet fever due to contact infection, the age distribution usually bears no definite relation to the age distribution of the population, but is determined by the number of susceptible children.

There has been no great advance in the knowledge of these infections, but numerous papers have been published giving additional evidence in support of the very great value of Lancefield's grouping and Griffith's typing methods in the elucidation of many of the epidemiological problems associated with the *Str. pyogenes*, and have been largely devoted to the infection of wounds in hospital wards, and to the cross-infection of cases. As regards cow infections, Little (9) has found, by experiment, that,

when human strains of beta-haemolytic streptococci were introduced via the teat canals into the udders of four cows, a mastitis was induced in eleven quarters. The infection persisted in five, while in the other six quarters recovery took place at varying intervals. These experiments, together with tests carried out with bovine strains, showed that, while human strains can infect the udder, they do not persist in the tissues like bovine strains.

Raw-milk samples have been examined by Gunnison *et al.* (10) for all types of haemolytic streptococci. From 444 such samples they were able to obtain organisms which could be classified by the Lancefield method, as follows:

Group A strains:	3 samples
Group B strains:	125 samples
Group C strains:	1 animal type
Group D strains:	2 human types
Group E strains:	2
Group G strains:	6
Group H strains:	1

Group A strains, potentially dangerous for human beings, were therefore only recovered from less than 1 % of samples.

In the report of the chief medical officer of the Ministry of Health for 1938 (11), and in a paper by Samson (12), there is described a small localized outbreak of scarlet fever, apparently caused by the consumption of infected raw milk in the borough of Romford. Eighteen cases were notified, and of these five were residents and workers at a farm which supplied the milk to the other thirteen cases. Pasteurization of the supply was promptly instituted and the outbreak ceased, but veterinary inspection failed to disclose any pathological lesion in the cows. Again, in the 1938 report of the Department of Health for Scotland (13), an outbreak of scarlet fever, due to a milk infection, is recorded. This outbreak of 161 cases occurred in the county of Ross and Cromarty, and was due to the milk from one farm. Henningsen & Ernst (14) give a report of a milk epidemic of scarlatinal angina, comprising 118 cases of scarlet fever and about fifty cases of sore throat. The infection was spread through contamination of the milk by a milkmaid suffering from a purulent otitis media after an attack of non-recognized scarlet fever. By cultural and serological methods, *Str. pyogenes*, group A (Lancefield), type 3 (Griffith), was isolated from 108 of these patients, and from pus from the ear of the milkmaid. The cows, and the milk obtained from them, were examined twice at an interval of 6 days, and no abnormality was found. Inquiry into the conditions under which the milk was transported from the producer via the dairy to the consumers, and information concerning the distribution and consumption of the milk, confirmed in all details the assumption as to the causal connexion between the otitis media in the milkmaid and the outbreak of the epidemic. This epidemic furnishes an example of transmission of the infection from man, directly through the milk, to a fairly large number of consumers without any intermediate mastitis in a cow.

Staphylococcal food poisoning

Considerable attention continues to be paid to the study of staphylococcal toxins and, in particular, to the enterotoxin. Dolman & Wilson (15) have carried out further researches on the production of staphylococcal enterotoxin and its detection by the kitten test, and they describe a semi-solid agar medium which gives good yields. After the filtrate is recovered, it is treated by dilute acetic acid and by boiling to destroy the thermolabile toxins, and thereafter is used for the intraperitoneal injection of kittens. For the test they suggest the use of three kittens, 6–12 weeks old; one should receive 3 ml. and another 1 ml. of the filtrate, while the third, the

control, should be given 3 ml. of the uninoculated broth. A positive enterotoxin test is indicated by symptoms of lassitude, weakness, unsteadiness, and strong peristaltic movements of the intestine producing vomiting and diarrhœa. Large amounts of a powerful enterotoxin may cause death. Again, Bayliss (16) has studied the mechanism of vomiting produced by staphylococcal enterotoxin on young and adult cats under various experimental conditions. The author concluded that the action of *Staphylococcus* enterotoxin on peripheral sensory structures is of greater importance in the initiation of emesis than direct action on the vomiting centre.

The part played by animals of the bovine species in the dissemination of staphylococcal infected milk has again received attention. Plastridge *et al.* (17) have published an extensive paper on infectious bovine mastitis, due to staphylococci. These workers point out that staphylococci are of interest to the dairy industry, since they are an important cause of mastitis, since they lower the quality of the milk by producing a high leucocyte and bacterial count, and since, under certain conditions, some strains are capable of producing substances which are toxic to man. After a careful examination of the whole problem, the authors concluded that an enterotoxin-producing type of *Staphylococcus* can be recovered from mastitis in cows, that there is no satisfactory cultural test for differentiating potential food poisoning from non-food poisoning types, and that environmental conditions must be such as to allow the rapid growth of staphylococci for the production of enterotoxin. It is further suggested that this type of food poisoning can be largely eliminated by immediate refrigeration, but that once the toxin has been elaborated in the milk, neither pasteurization nor boiling will eliminate this product, though the staphylococci will be killed off. Again, Blair (18) has reviewed certain aspects of recent investigations on staphylococci, and, in particular, he has summarized the literature dealing with exotoxins, differentiation of pathogenic and non-pathogenic types, and serological classification and mode of infection. The author points out the importance of staphylococci from bovine mastitis in food poisoning, and believes that contamination from human sources plays a minor part in the epidemiology of the disease. As regards the identification of pathogenic types by in vitro tests, the author states that the evidence in the literature and his own experience have led him to conclude that the coagulase test alone is a sufficient indicator of the pathogenic potentiality of staphylococci, and that the fermentation of mannitol is a valuable confirmatory one.

The clinical symptoms associated with staphylococcal food poisoning are, a sudden onset 2–4 hr. after the ingestion of food, of nausea, vomiting, dizziness, some abdominal pain, slight diarrhœa, and sometimes prostration; the patient, however, makes a rapid recovery, and is usually completely well within 24 hr. Only one fatal case has, so far, been reported, and several new epidemics have recently been described. Further, Shaughnessy & Grubb (19) have encountered a number of milk-borne staphylococcal food-poisoning infections associated with milk from small herds and supplied to small communities. They found in all these outbreaks cows suffering from staphylococcal mastitis, but the milk was normal in appearance and taste. They believe that the infrequency of such outbreaks in large communities is due to the dilution of the affected milk by bulking. Finally, the authors found that the most reliable method of detecting enterotoxin is to feed sterile culture filtrates to human volunteers. Again, Pikkarainen (20) described two outbreaks of staphylococci food poisoning. The first involved twenty-six individuals, and the symptoms were those usually associated with this infection. The common food factor was sugared cake containing a cream layer, and from the cream a profuse growth of *S. aureus* was obtained. The second outbreak caused illness in only nine individuals, and the common factor, which was again cream cakes, contained numerous types of staphylococci, including *aureus*. No tests on culture filtrates were carried out. Hackler (21) recorded a small outbreak amongst students at the Oklahoma Agricultural and Mechanical

College. In all, twenty-nine individuals from eleven different residences were involved. Potentially toxic staphylococci were repeatedly isolated from the milk supply, which had been efficiently pasteurized. Bacteriological examination of workers at the pasteurizing plant showed that three employees were carriers of possible enterotoxin-producing staphylococci in either the throat or nose. The author presumed that contamination of the milk had occurred after pasteurization during the bottling and capping operations. Other outbreaks have been described by Roberts (22) and by Roberts & Wilson (23). Cream cakes sold from a certain bakery in Ontario produced outbreaks of staphylococcal food poisoning in March, and again in May 1938. In March 1939, there again occurred a further outbreak of food poisoning, involving 140 cases in sixty-three families. The symptomatology of the illness pointed to a staphylococcal infection, and investigation showed that the common food factor was again cream-filled cakes obtained from the bakery which had already caused the previous outbreaks. *S. aureus* was isolated from the cream filling, and from throat and nose cultures of five employees at the bakery, including the employer. The staphylococci were all alike in their cultural reactions, and produced an enterotoxin which was demonstrable by the kitten test.

Two outbreaks in which the symptomatology suggested the *Staphylococcus* enterotoxin as the causative agent, but in which no certain proof was obtained, have been described. A brief account of the first is given in the report of the chief medical officer of the Ministry of Health for 1938 (24). This outbreak occurred in a school, and involved sixty cases. The second epidemic occurred in Australia, and Dale (25) gives a description of this large outbreak of food poisoning, involving 197 individuals, in which the symptoms were sudden onset, nausea, vomiting, abdominal pain, and diarrhœa, usually with rapid recovery in 24 hr., and there were no deaths. No less than 140 cases occurred during a period of 3 days, and the cases were distributed over ten institutions. The only common factor was the milk supply, which was obtained from one farm mainly, but was supplemented from eight different sources. The bacteriological examination of milk, faeces, vomit and blood sera gave entirely negative results.

Tuberculosis

The danger of supplying human beings with milk which contains tubercle bacilli is universally recognized, yet in many countries the administrative methods adopted to eliminate this source of human ill-health are slow, cumbersome and ineffective.

A number of papers have appeared on the value of certain technical procedures used in connexion with the identification of tubercle bacilli. The method first elaborated by Cowan & Maddock to facilitate the direct examination of films made from milk deposits, namely, the localization of epithelioid and giant cell groups, and the search for tubercle bacilli in these, has found great support. Ziegler (26) reports the finding of typical cell groups in 313 out of 861 cases in which tubercle bacilli were also found at the same time, and in 218 cases in which they were found later. Pallaske (27) suggests that the additional procedure of embedding the deposit in paraffin, and the cutting of serial sections, would give even more satisfactory results. Gloyne (28) states that of the three possible methods of examining milk, namely, direct film examination of the centrifuged deposit, cultivation, and guinea-pig inoculation, the last yields by far the best results. It gives ten times more positive results than direct microscopic examination, while cultural methods are handicapped by the presence of saprophytic spore-bearing aerobes. The guinea-pig test not only gives the highest percentage of positives, but it is also the only test which can be used as absolute proof in a court of law. As regards the identification of tubercle bacilli in human specimens, two papers confirm the value of the guinea-pig test. Whitehead (29) examined 215 specimens from various types of human disease for tubercle bacilli. The methods

used were direct examination, concentration, culture on four types of media, and guinea-pig inoculation. The authors concluded that Petragnani's malachite-green medium was the most suitable, but the results of culture did not warrant its substitution for guinea-pig inoculation. Culture and guinea-pig inoculation combined give the highest degree of reliability. Again, Hoyt *et al.* (30) carried out an extensive series of parallel cultures and guinea-pig inoculations with human materials for tubercle bacilli, with the following results:

Guinea-pigs positive	Cultures positive	Both cultures and guinea-pigs positive	Guinea-pigs positive, cultures negative	Guinea-pigs negative, cultures positive
301	242	198	103	44
87 %	70 %	57 %	30 %	13 %

These results indicate that the animal inoculation method is the most sensitive, although neither animal inoculation nor culture is entirely reliable.

A new line of approach to the differentiation of human and bovine tubercle bacilli has been suggested by the work of Griffith (31, 32), when he found that voles (*Microtus agrestis*) are highly susceptible to bovine bacilli, and that they can also be infected with human tubercle bacilli, though apparently with less certainty than with bovine bacilli. Wells (33) has amplified this, and has found that the intraperitoneal injection of at least 1 mg. of moist culture is necessary to produce demonstrable disease within 1 month. Further, his work indicates that a dose of 0·001 mg. moist weight tubercle bacilli injected intraperitoneally into voles should distinguish unfailingly between human and bovine tubercle bacilli in 1 month. Griffith (34, 35) has carried out a further extensive series of experiments, and his results are as follows: The vole is apparently susceptible to infection with strains of all four types of tubercle bacilli, and, as previously reported, parenteral injection of the bovine type gives rise to generalized progressive tuberculosis, with marked evidence of caseation. When human tubercle bacilli are injected, they can multiply in the tissues, but produce little macroscopic evidence of disease, while the avian tubercle bacillus produces results similar to the human type. A further important observation was that certain bovine strains, which, when injected into rabbits, only showed a reduced virulence, and were consequently difficult to distinguish from human types, produced, when injected into voles, the characteristic pathological changes. Finally, the author concludes that the vole may be of great assistance in determining the type of a strain of tubercle bacillus which, on cultural examination, has bovine type characteristics, and on rabbit inoculation produces equivocal results.

Numerous papers again record the part played by the bovine type of tubercle bacillus in the production of pulmonary and non-pulmonary lesions in human beings. Table 5 summarizes the results of recent investigations into pulmonary disease due to the bovine type. The findings recorded by different workers show wide variation, but the highest incidence is found in cases in the country districts of north-east Scotland, and the lowest incidence in Rome. The figure for Scotland as a whole is fairly high, but not so high as that recorded by Lange (36) for Germany. Munro (41) suggests that, despite these alarming figures, there is probably no real increase in the number of bovine cases, and that the relative increase is due to a steadily decreasing number of cases infected with the human type. There is little evidence to show that any great proportion of these cases is due to direct contact with infected cattle, the main source of infection being undoubtedly due to tuberculous milk. In a few instances, members of the same family have been found to have a bovine-type infection, and it is presumably possible for an infected mother to infect her children. As regards non-pulmonary bovine tuberculosis, the observations made have not been numerous, and Table 6 summarizes the findings obtained. Price (44) points out

that in his series of cases, all patients showing a bovine type of tubercle bacillus contracted the infection outwith the city of Toronto, where the entire milk supply is efficiently pasteurized. In Mushatt's (45) series of cases, ten out of fourteen strains from cases of cervical disease, four out of twenty-two in bone and joint lesions, and three out of twelve from meningitis cases, were organisms of the bovine type. The findings of Meyer *et al.* (46) for northern France show an extremely low rate of infection due to tuberculous milk in osteo-articular and glandular lesions.

Table 5. *Incidence of bovine pulmonary tuberculosis*

Worker	Country	Total specimens examined	No. showing human T.B.	No. showing bovine T.B.	% positive bovine T.B.
Lange(36)	Germany	171	158	13*	7·6
Petragnani(37)	Rome	564	560	4	0·7
Gernez & Crampon(38)	Northern France	266	263	3	1·1
Ruys(39)	Holland: Cities	1075	1053	22	2·0
	Counties	845	806	39	4·6
	Both	1920	1859	61	3·1
Carmichael(40)	Uganda	283	279	4	1·4
Munro(41)	Scotland (Glenlomond Sanatorium)	1061	1010	51†	5·0
Griffith & Smith(42)	Scotland: Counties	540	491	49	9·1
	North-East: Cities	422	413	19	4·4
	Total	962	904	68	7·0
Griffith(43)	Scotland: middle and south	484	453	31	6·0
	All Scotland	2507	2367	150	5·9

* Four containing human and bovine. † One mixed.

Table 6. *Non-pulmonary tuberculosis. Incidence of bovine type in human infection*

Author	Country	No. of specimens of pus, etc.	No. positive for bovine type	% positive
Price(44)	Canada (Toronto)	500	48	9·6
Ruys(39)	Holland	649	149	22·6
Mushatt(45)	Eire	50	17	34·0
Meyer *et al.*(46)	France	250	8	3·2

Salmonella infections

(1) *Enteric fevers*

Certain progress has been made in diagnostic methods, and the detection of Vi agglutinins in the sera of suspected carriers may serve as a helpful indicator of an actual carrier condition. Strains of *Salmonella typhi* isolated from different epidemics cannot be classified into groups or types by the usual serological methods. Craigie & Yen (47), however, devised a method of typing by means of an anti-Vi phage, which could be developed by serial passage, to act selectively on certain strains. By this means, types of *S. typhi*—A, B_1, B_2, C, D_1, D_2, E, F, G, H and J—have been identified. The authors found that, in various outbreaks, the strains isolated from the cases, and from the carrier causing the outbreak, could be correlated. By this means, when, on the examination of suspects, several carriers are discovered, it is possible to say which one is actually responsible for the outbreak, since all strains from a particular epidemic conform to one type. Boyd (48) confirmed and extended the findings of Craigie & Yen.

Few epidemics of enteric fever have been reported as being due to milk. An unusual method of contamination is described by Neveu (49) in which the milk vessels were washed in a tank where the clothes of the milk dealer's wife, who had suffered from typhoid fever, were also rinsed. Again, Wilson (50) gave particulars of an outbreak of typhoid fever which occurred in Belfast in 1935. Here it was found that a stream, which passed through the yard of the dairy which distributed the infected milk, had become contaminated by sewage, which had escaped from a broken pipe. When the water from the stream was examined bacteriologically, no less than 300 typhoid organisms per ml. were found. The method by which the milk was actually infected could not be discovered, but as a result of this infection over 100 cases were admitted to hospital. Lemaire (51,52) reported a large outbreak of typhoid fever in Algiers, extending over a period of 5 months, and involving 370 individuals and causing no less than ninety-nine deaths. Inquiries suggested that the vehicle of infection was ice cream, and the owner of the confectionery business was found to be a carrier, so it was presumed that he was the source of infection. The annual report of the Department of Health for Scotland for 1938 (53) records a large outbreak of typhoid fever in the town of Hawick. The total number of cases, including a carrier, amounted to 107, of whom 103 resided in Hawick. Only five secondary cases were reported, and only five deaths occurred. The incubation period of the disease was extremely variable, and the onset very insidious, so that it was impossible to determine accurately the date of infection. The age and sex distribution, and the slow rise of the epidemic, contra-indicated a water-borne infection, so also the geographical distribution. Eventually it was concluded that the vehicle of infection was cream in cream buns, and a thorough bacteriological examination of the staff of the creamery showed that one female worker was a carrier. Finally, Landau (54) reported an epidemic of sixty-seven cases of typhoid fever in South Africa. Fifty-two of the cases had received their milk supply from one particular dairy. The dairy supplied forty-eight families, and 30 % of the individuals receiving the affected milk developed typhoid fever. Fourteen of the cases died, giving a case mortality rate of 21 %. There was no definite evidence of how the milk supply was actually infected.

(2) *Food-poisoning infections*

Attention has already been called to infection in cows with *Salmonella* type *dublin*. The report by Conybeare & Thornton (55) contains a summary of the literature on this subject, together with an account of an outbreak due to raw milk. Tulloch (56) described two outbreaks of enteritis, which were proved to be due to *S. dublin*. The first occurred in Dundee in 1926, and involved 373 individuals in 192 households, while the second took place in 1927. One hundred cases from thirty-four families were proved to be suffering from this infection, but it was estimated that at least 280 individuals were actually involved. The age distribution of these cases was as follows:

	First epidemic	Second epidemic
Under 5 years	59	6
5–15 years	42	14
Over 15 years	272	80
Total	373	100
No. of families affected	192	34

In both outbreaks the sources of infection were determined, and proved to be cows which had indubitably suffered from a septicaemic infection, as the etiological agent was recovered from milk and from various other organs obtained from the animals when slaughtered. Bartel (57) collected numerous *Salmonella* types isolated from

domestic animals—calves, cattle, pigs, horses and sheep—and the strains isolated from bovine specimens were classified as follows:

Salmonella types

	S. dublin	*S. typhi murium*	*S. enteritidis*	*S. enteritidis* var. *rostock*
Calves	1027	99	8	9
Cattle	253	120	8	1

	S. morbificans bovis	*S. para-typhosus* B	*S. suipestifer*
Calves	0	0	0
Cattle	1	2	1

It will thus be seen that, in bovine *Salmonella* infections, the *dublin* type of organism predominated over all others. Of the 253 infections in adult cattle, seventy-five were found among chronic carriers, and 158 among emergency slaughtered animals that were suffering from inflammation of various viscera. In human infections *S. typhi murium* infections are predominant.

Dysentery

The serological classification of the Flexner dysentery bacilli has been recently reinvestigated by Boyd (58). This worker has found that the Flexner types V, W, X and Z of Andrewes and Inman are definite serological entities, but that the original

Table 7. *Flexner dysentery bacilli. Boyd*

Type	Lactose	Glucose	Mannitol	Dulcite	Serological characteristics	Where found
V, W, X, Z, Andrewes & Inman	—	A	A	—	All distinct types	World-wide distribution
Type 103	—	A	A	—	Distinct type	India; England
Type 119	—	A	A	—	Distinct type	India; West Africa
Type 88 (33 %)	—	A	A	—	Serologically identical	India
Type 88 (66 %)	—	A	A	A (late)		India
Newcastle bacillus	—	Ag	—	Ag (late)		Great Britain and elsewhere
Manchester bacillus	—	Ag	Ag	Ag (late)		Great Britain and elsewhere

Y type could not now be regarded as such. Strains of this Y type were found to consist solely of non-specific antigens, and are composed of a mixture of the other four types. Boyd has also added other organisms to the Flexner group, namely, type 88, which serologically is identical with the Manchester and Newcastle strains, but differs from them in biochemical reactions, and types 103 and 119, which give biochemical reactions similar to the Flexner strains V, W, X and Z. Table 7 summarizes the main characteristics of this group.

Only one report of a milk-borne outbreak of dysentery appears to have occurred during the past two years. Trimble & Brothwood (59) described an outbreak of Sonne dysentery in which the milk was supplied to twenty-four households but only twelve families were affected, and although eighty-six persons were exposed only twenty-one became ill. The epidemic ceased as soon as the distribution of the supply was prohibited, thus making it clear that the outbreak was milk-borne, but it was not clear as to the manner in which the milk became contaminated.

Brucella infections

Much work continues to be done on the control of *Brucella* infection in domestic animals. The elimination of reactors from herds of dairy cows, and attempts at immunization against infection, have been the two main lines of approach to this

problem. So far as human beings are concerned, this disease is only sporadic in its occurrence in this country and America.

There has been no recent technical advance in methods designed to assist in the isolation of *Brucella* organisms from milk, or milk products, or in the methods used to distinguish the various members of the group. Occasional dissatisfaction continues to be expressed with the agglutination test in the diagnosis of human infections: thus, Badoux (60) believes that this test is not so sensitive as the complement-fixation method, but he based his conclusions on the results of examination of some sixty-two specimens, in which fourteen gave a positive complement-fixation test, and seven a positive agglutination reaction. The survival of *Br. abortus* in milk and milk products has been studied by Liechtenstein (61). He found that the organism may remain alive for many days, after the production of lactic acid, if this does not exceed 22 SH°. If, on the other hand, the lactic acid rises to 34 SH°, or over, then it dies in 2 days. The whipping of cream had no appreciable effect on the survival of the organism, while in skim milk at 37° C. organisms increased for the first 2 days and thereafter diminished, but when the skim milk contained specific agglutinins the increase was much less. Menton (62) examined 1000 milk samples, and found whey agglutinins in 34·5 %, but only 29·1 % of these samples contained living *Br. abortus*, whereas 9·1 % of samples devoid of agglutinins contained *Br. abortus*. Fifty samples of butter gave negative results. Menton has also shown that when infected material is inoculated into guinea-pigs, the best time to demonstrate the presence of agglutinins is 6 weeks after inoculation. He believes, also, that a precipitation test might be more useful than the demonstration of agglutinins, and for this purpose prepares an antigen from a 72 hr. live agar culture by extraction with *N*/20 HCl, and after centrifugation NaOH is added, the precipitate so formed is discarded and the supernatant fluid used in the test.

As regards the incidence of the disease in human beings in England, the Annual Report of the Chief Medical Officer for 1938 (63) gives the following yearly incidence:

1926	2	1933	64
1927	6	1934	58
1928	5	1935	39
1929	17	1936	51
1930	30	1937	56
1931	42	1938	38
1932	66	Total	474

In the report, also, there appears a note by MacKenzie (64) on the incidence of undulant fever in New Zealand. This disease was first made notifiable there in 1930, and for the period 1928–38, 196 cases were reported, and of these, 139 were males and fifty-seven females, showing once again the great preponderance of male cases to female. Again, sixty-five of the patients were farmers, or farm workers, eleven were wives or children of dairy farmers, and four were engaged in other occupations, but milked cows. In addition, the series included four slaughtermen, one veterinarian, and one bacteriologist working with *Br. abortus*, while five individuals in the series died. The author further suggests that contact with infected cows is a common source of infection, and is perhaps the most dangerous. As regards infection by the consumption of milk, the incidence appears to average about eleven cases per annum, though only a small proportion of the milk is pasteurized. From Germany a report (65) states that during 1936 and 1937, 587 and 586 cases respectively were treated in various hospitals, and of these, 429 and 395 occurred in Prussia, and those affected were mainly agricultural workers. The chief source of infection was presumed to be raw milk and raw milk products. Only seven deaths were reported as having occurred in a total of 1173 cases, giving a mortality rate of 0·6 %. Elkington *et al.* (66) reported

an interesting mild epidemic of undulant fever in a school of 400 boys. Two definite cases occurred 3–5 weeks after the beginning of the term, and twenty-six suffered from a transient illness, with fever, headache, malaise, and listlessness, and of these, ten gave positive agglutination tests ranging from 1/20 to 1/100. The sera from 17 boys, who showed no signs of illness, were also examined, and five gave positive agglutination tests with *Br. abortus* in titres ranging from 1/20 to 1/1000. *Br. abortus* was recovered from the raw milk supply. The authors concluded that the raw infected milk produced clinical, subclinical and latent undulant fever in some 30 % of the boys, and emphasized the protean nature of the symptomatology.

Virus infections

Little evidence has, so far, been forthcoming on virus diseases being transmitted through the agency of milk. Human beings are apparently relatively insusceptible to the virus infections of bovines, and perhaps even when milk is contaminated from human sources it is not a good vehicle for the transmission of such diseases.

From time to time reports appear on cases of foot and mouth disease in human beings, but the evidence of infection through milk is usually not very conclusive. Joltrain (67) describes such a case, and attributes the infection to the ingestion of milk from an infected cow, and also white cheese. Fischer (68) has also described the disease in human beings, and suggests that one source of infection may be infected milk. In some countries, the possibility of the transmission of rabies through milk has been suggested, but Graham & Dunlop (69) have examined this problem experimentally, and have shown that young rabbits could be nursed by a rabid doe up to the time of the doe's death without themselves acquiring the disease.

Miscellaneous infections

From time to time, reports are published incriminating unusual bacteria as the cause of food poisoning. Two papers have recently appeared in which cheese was the vehicle of infection, and strains of *B. coli* were isolated from this milk product. Florentin (70) describes three minor outbreaks of food poisoning due to cheese, the first in which three out of four members of a family were taken ill, the second in an adult male, and the third in which fourteen individuals developed an illness. The incubation period was short, and the illness comparatively mild. From the various types of cheese involved, the author isolated *B. coli* strains. He suggests that the toxin of *B. coli* may be the cause. Again, Schaede (71) reported an outbreak of food poisoning, which involved individuals in four camps, and in which the common food substance was Limburger cheese. The illnesses were characterized by a short incubation period, and by symptoms indicative of food poisoning. Bacteriological examination failed to show any *Salmonella* types, but showed *B. coli*. Mice fed with the cheese were unaffected, but one strain of *B. coli*, when grown in broth for 5 days and then fed to mice, caused diarrhœa and death. The same cultures, when heated to 56° C. for 1 hr., were harmless when fed to mice.

J. SMITH

City Hospital Laboratory
City Hospital
Aberdeen

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